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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

RESPIRATORY METABOLISM AND THE ACTIVITIES OF CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE DURING THE EMBRYONIC DEVELOPMENT OF THE JAPANESE BEETLE, POPILLIA JAPONICA NEWMAN [1]

DANIEL LUDWIG AND MICHEL WUGMEISTER
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TWO FIGURES

Fink ('25) studied the respiratory metabolism of the Japanese beetle egg at temperatures between 21° and 24°C. He found that at these temperatures, the respiratory rate remained low during the first 6 days and then increased rapidly. He called the first 6 days of the embryonic period the "formative period." Rothstein ('52) studied biochemical changes occurring during the embryonic development of this insect at 30°C. and made a few observations on the rate of oxygen consumption. At this higher temperature the formative period lasts 4 days (newly-laid to 3-day egg). Rothstein found that glycogen serves as the source of energy during the period when the rate of oxygen consumption is low, and that free fat is used during the remainder of the embryonic period. The first 4 days of embryogenesis at 30°C. are also characterized by a rapid increase in weight due to the imbibition of water, the weight then remaining approximately constant until the time of hatching. Ludwig and Rothstein ('52) found a rapid increase in the nitrogen content of the water-

[1] This investigation was supported in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-444.

insoluble fraction of the Japanese beetle egg during the first 4 days of embryonic development at 30°C., followed by a slow and irregular increase during the remainder of the embryonic period. They believe that the increase in the insoluble nitrogenous fraction indicates the formation of structural proteins. They suggest that, at this temperature, a possible change in the mechanisms involved in embryogenesis may occur after the fourth day of embryonic development and that this change may involve the cytochrome system.

Gese ('53) studied the embryology of the Japanese beetle using carefully timed eggs incubated at 30°C. He found that at this temperature, the first 4 days actually constitute a formative period during which the germ band develops and grows around the yolk dorsally, and the appendage buds and internal organs are formed. Between the fourth and fifth days, the process of blastokinesis occurs. During this process, the embryo changes its position by first shortening, and then flexing ventrally so that the anterior and posterior ends almost meet beneath the germ band to form the typical scarabeiform larva. Further development then consists essentially of the growth and differentiation of structures already present.

The present studies were undertaken to determine whether the shifts in respiratory metabolism, sources of energy, and other biochemical changes, as well as the morphological changes which occur at the fourth day of embryonic development, may be correlated with the activities of the respiratory enzymes, cytochrome oxidase and succinic dehydrogenase.

MATERIAL AND METHODS

The eggs used in these experiments were collected in the laboratory each morning, so that, when obtained, they were always less than 24 hours old. They were then placed, 100 to a box, in 1-ounce metal salve boxes containing moist soil and incubated at 30°C. Determinations were made on the rate of oxygen consumption, on respiratory quotients, and on the activities of cytochrome oxidase and succinic dehydrogenase for each day of embryonic development.

Oxygen consumption was measured at 30° C. on groups of 50 eggs, using constant volume Warburg manometers, according to the procedure outlined by Umbreit, Burris, and Stauffer ('45). For these determinations, 0.2 ml of 10% KOH was placed in the central well of the manometer vessel for the absorption of the carbon dioxide. A small piece of filter paper was folded and placed in the KOH to increase its surface area. In determining the rate of carbon dioxide production, the eggs were placed in the manometers without the KOH. Respiratory quotients were calculated from the values obtained from these two groups of determinations.

The activity of cytochrome oxidase was determined on groups of 20 to 50 eggs, depending on weight, according to the method of Cooperstein and Lazarow ('51). Details regarding the preparation of the various solutions and methods for preparing the material are given by Ludwig ('53). Dilutions of the homogenate were always made on a weight basis.

The activity of succinic dehydrogenase was also determined on groups of 20 to 50 eggs. Details of the procedure are given by Cooperstein and Lazarow ('50). Dilutions for the determination of succinic dehydrogenase were also made on a weight basis.

OBSERVATIONS

The rates of oxygen consumption, expressed as micro-liters of oxygen per 50 eggs per hour, for each day of embryonic development are shown in figure 1. Each value represents an average of at least 10 determinations. The rate in newly-laid eggs is low, averaging 10.85. It increases to an average of 23.27 in the 1-day egg and remains at approximately this level through the 3-day egg. It then increases steadily until the time of hatching. The value (29.19) for 4-day eggs is significantly higher than that (25.16) for 3-day eggs, since the difference between these values is more than twice its standard error. The respiratory quotients during embryonic development decrease from a relatively high value of 0.89 in newly-laid eggs, to 0.75 in eggs just before hatching.

The activities of cytochrome oxidase for each day of embryonic development, expresed as Δ log [Cy Fe $^{++}$] /minute for homogenates in a dilution of 1:1,000, are shown graphically in figure 2. Cytochrome oxidase activity is high in newly-laid eggs and decreases rapidly reaching a low value in 4-day eggs. Since the Japanese beetle egg increases in weight during this same period from an average of 0.87 to 2.44 mg

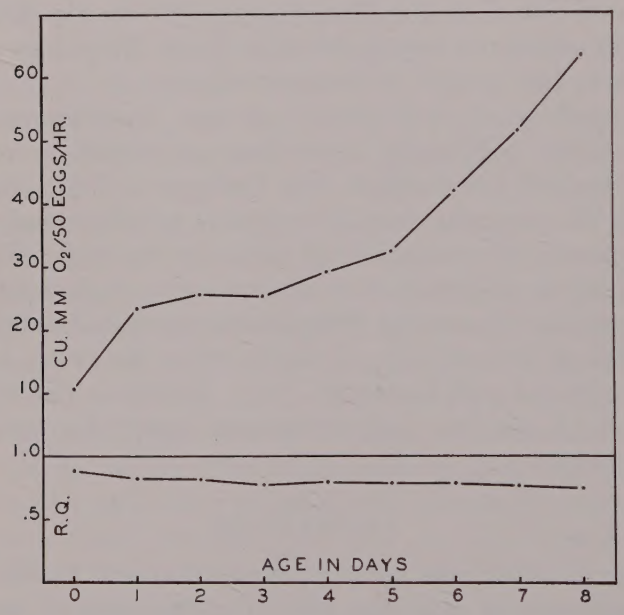

Fig. 1 Respiratory metabolism during the embryonic development of the Japanese beetle. O, newly-laid egg.

due to the imbibition of water, and since all homogenates were diluted in a weight basis, it is probable that the decrease in activity is associated with a dilution of the enzyme. The first part of the cytochrome activity curve is the reciprocal of the weight curve for the Japanese beetle egg. However, beginning with the 5-day egg, the activity of cytochrome oxidase increases rapidly until the end of the embryonic period, although weight remains approximately constant. It thus appears that

cytochrome oxidase activity remains at a constant low level during the first 5 days of the embryonic period and then increases during the remainder of embryogenesis.

The activities of succinic dehydrogenase, expressed as Δ log [Cy Fe $^{+++}$] /minute for homogenates in a dilution of 1:1,000 are given in figure 2. The activity of this enzyme shows the

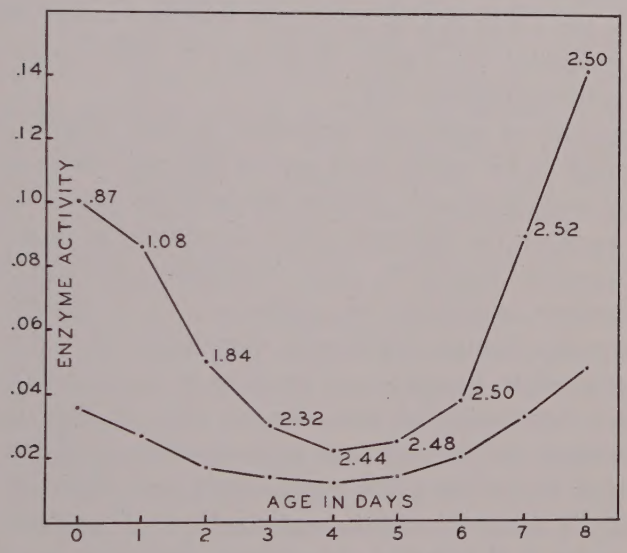

Fig. 2 Enzyme activity during the embryonic development of the Japanese beetle. Upper graph, cytochrome oxidase activity, expressed as Δ log [Cy Fe $^{++}$] /minute for homogenates in 1:1,000 dilution; lower graph, succinic dehydrogenase activity, expressed as Δ log [Cy Fe $^{+++}$] /minute for homogenates in 1:1,000 dilution; O, newly-laid eggs. Numbers on upper graph are average weights, in milligrams, of eggs.

same series of changes as those described for cytochrome oxidase, except that the values are considerably lower. At the beginning and at the end of the embryonic period, the activity of succinic dehydrogenase is approximately one-third that of cytochrome oxidase. However, during the middle of the embryonic period, it is approximately one-half that of cytochrome oxidase.

DISCUSSION

The relatively low rate of oxygen consumption observed for the first 4 days (newly-laid to 3-day egg) of the embryonic period and the rapid rise after the fourth day at 30°C., are in agreement with the results of Fink ('25). He found that at 21° to 24°C., the low respiratory rate for the egg of the Japanese beetle continued for at least 6 days. The increased rate described for the 1-day egg as compared with the newly-laid egg, was not reported by Fink. However, it was observed by Rothstein (unpublished work).

The respiratory quotients reported in this paper, high at the beginning of the embryonic period and gradually decreasing during embryogenesis, follow the general trend observed by many workers for the embryonic development of a variety of animal forms (Needham, '42). Needham used the decreasing respiratory quotients as evidence for a succession of energy sources during embryonic development, with carbohydrate preceding protein and protein preceding fat. Rothstein ('52) demonstrated that, in the egg of the Japanese beetle, glycogen utilization does precede the use of fat. However, neither Rothstein ('52) nor Ludwig and Rothstein ('52) were able to demonstrate the utilization of protein as an energy source in the egg of this insect. The high respiratory quotients obtained during the first few days of the embryonic period may be correlated with Rothstein's observation that glycogen serves as the energy source during this period. The low rate of oxygen consumption observed during the first 4 days further supports the suggestion, previously made by Ludwig and Rothstein ('52), that a portion of the glycogen breakdown is anaerobic. The lower respiratory quotients reported for the last few days of the embryonic period are in accord with the observation of Rothstein ('52) that after the fourth day, fat is the main source of energy.

The suggestion that the decrease in the activities of cytochrome oxidase and succinic dehydrogenase during the first part of the embryonic period of the Japanese beetle may be

caused by a dilution of the enzyme, is in agreement with the work of Moog ('52). She found that in the chick embryo the relative activities of cytochrome oxidase and succinoxidase fall during the first three days in association with the imbibition of water.

A comparison of the graph for oxygen consumption (fig. 1) with those for enzyme activity (fig. 2) shows that respiratory metabolism increases in the 4-day egg before the activities of cytochrome oxidase and succinic dehydrogenase have started to rise. After this stage, the rate of respiratory metabolism appears to be directly correlated with the increase in the activities of both respiratory enzymes. This observation is similar to that of Allen ('40) who studied the relationship between cytochrome oxidase and respiratory metabolism in the egg of the grasshopper, *Melanoplus differentialis*. Allen found that the rate of oxygen consumption increases throughout post-diapause, whereas the oxidase is present during the early part of this period in amounts not significantly different from those occurring in the diapause egg. Later, it increases in a direct relationship to the rate of oxygen uptake. In the Japanese beetle, the higher rate of oxygen consumption beginning with the 4-day egg may be associated with the phenomenon of blastokinesis which Gese ('53) observed to be complete at 120 hours of incubation at 30°C.

The differences in the activity values for cytochrome oxidase and succinic dehydrogenase in the Japanese beetle egg are essentially the same as those described for mammalian tissue. Schneider ('46) reported that in rat liver cells, succinic dehydrogenase has an activity approximately one-third of the activity of cytochrome oxidase. This ratio is the same as that found in the present experiments for the first three days and the last two days of the embryonic period. However, Albaum, Novikoff, and Ogur ('46) found that in the developing chick embryo, the cytochrome oxidase/succinoxidase ratio begins at a peak of about 28 to 1, and reaches a constant value of 5 to 1 after the fifth day.

SUMMARY

1. Eggs of the Japanese beetle were collected daily and incubated at 30°C. Determinations were made on the rate of oxygen consumption, on respiratory quotients, and on the activities of cytochrome oxidase and succinic dehydrogenase for each day of embryonic development.

2. The rate of oxygen consumption, expressed as microliters of oxygen per 50 eggs per hour, is low, 10.85, in newly-laid eggs. It rises in the 1-day egg to 23.27 and remains at this level for the next two days. From the fourth day the rate increases steadily, reaching a high value of 63.4 at the time of hatching.

3. Respiratory quotients decrease during embryogenesis from an initial value of 0.89 to a pre-hatching value of 0.75.

4. Cytochrome oxidase activity is high in newly-laid eggs. It falls rapidly reaching a low value in the 4-day egg. This decrease in activity is associated with a dilution of the enzyme since the egg increases in weight during this period from 0.87 to 2.44 mg, due to the imbibition of water. Beginning with the 5-day egg, the activity of cytochrome oxidase increases rapidly until the end of the embryonic period although weight remains approximately constant.

5. Succinic dehydrogenase activity follows the same series of changes as cytochrome oxidase, but at much lower values.

6. Respiratory metabolism begins to increase in the 4-day egg before the activities of the two respiratory enzymes show an increase, but after this stage, the respiratory rate and enzyme activities seem to be directly correlated.

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PHOTORECOVERY FROM ULTRAVIOLET — INDUCED PIGMENTATION CHANGES IN ANURAN LARVAE

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ONE FIGURE

Since Kelner's ('49) clear-cut demonstration of photorecovery or photoreactivation after ultraviolet radiation in microorganisms, the phenomenon has been described in other forms, both animal and plant. Blum and Matthews ('52) found it to occur in *Amblystoma* larvae, marking the first demonstration of photorecovery in a vertebrate. The present paper reports an investigation of effects of ultraviolet radiation on certain pigmentation changes, with special reference to the photorecovery phenomenon, in another vertebrate, the anuran larva.

EXPERIMENTAL

The animals used were larvae of *Rana pipiens* and *Rana catesbiana*. Similar results were obtained with both species. They were maintained in individual finger bowls in some experiments, in others two to a bowl, in tap water containing clumps of dry sphagnum moss. The temperature was kept at approximately 21.5°C. Their diet consisted of boiled spinach.

When exposed to ultraviolet radiation, the tadpoles were just barely covered with water. The radiation was supplied by an intermediate pressure mercury arc, which was monitored by a photoelectric cell. The characterization of the radiation and the method of measurement of dosage is described by Blum, Kirby-Smith, and Grady ('41). The effective radiation

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consisted of wave lengths of 0.313 μ and shorter; the intensity was 1.3×10^3 ergs per cm^2 per second, inclusive of these wave lengths.

Illumination with "visible" radiation was from 15 W fluorescent lamps (daylight type) having a spectral range from approximately 4000 A° to 7000 A°. The lamps were placed at a distance of about 15 inches above the top of the finger bowls. In experiments in which animals were kept in darkness, the bowls were wrapped in aluminum foil.

Single doses of ultraviolet radiation

Single sublethal doses of ultraviolet radiation of varying amounts were administered to 30 larvae of *Rana pipiens* and 30 *Rana catesbiana* tadpoles. The larvae were placed in groups according to length, which varied from 16 mm to 44 mm in the case of *Rana pipiens,* and from 45 mm to 85 mm with *Rana catesbiana.* They were subjected to single dosages of ultraviolet radiation of wave lengths 0.313 μ and shorter ranging from approximately 5×10^4 ergs per cm^2 to 5×10^6 ergs per cm^2 delivered in durations of from 40 seconds for the smaller dosages to slightly over an hour for the larger doses. Half of each group was subsequently illuminated with "visible" light as described above; the other half was kept in total darkness.

Within 2 to 4 days after irradiation, dark streaks of melanophores appeared on the dorsal surfaces of the animals which had been placed in the dark, an example of which appears at D in figure 1. The streaks, brown to black in color, were located medially and sometimes laterally, and extended anteriorly to the region of the eyes. Their extension posteriorly varied; in some cases streaks were observed on the tail. The gross configurations of the streaks were linear, branched in some animals, stellate in some, and amorphous with branching extensions in others. Generally the streaks were slightly elevated above the rest of the dorsal surface in a fold. Microscopically the streaks were found to consist of many epidermal melanophores of irregular sizes and con-

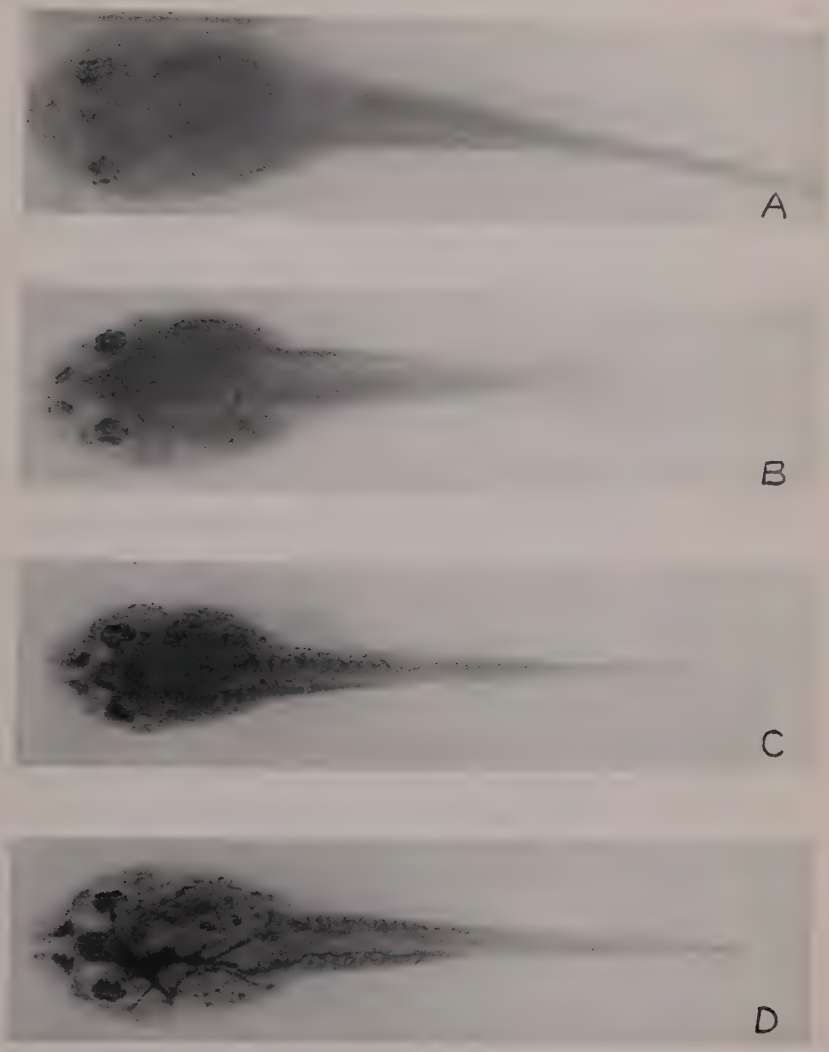

Fig. 1 Larvae of *Rana pipiens*. The larvae were from 38 to 42 mm long at the beginning of the experiment. The animals in A and B received no ultraviolet irradiation. The former was kept in "visible" light, the latter in total darkness. The animals in C and D were exposed to a single dose of ultraviolet radiation amounting to 2.7×10^5 ergs per cm^2 of wave lengths 0.313 μ and shorter, delivered in about $3\frac{1}{4}$ minutes. The specimen in C was placed in the light following irradiation, whereas the animal in D was maintained in darkness. The larvae are shown 6 days after irradiation. The streaking described in the text is clearly illustrated in D, but absent in the other animals.

figurations varying from amorphous structures with no processes to elongated ones with two or more processes. The streaks in *Rana catesbiana* contained a preponderance of the amorphous type melanophores, whereas in *Rana pipiens* both varieties were common. In the region of the streaks, these melanophore processes were in general considerably thicker, presumably due to an increased melanin content, than were those of the epidermal melanophores of normal tadpoles. There was a piling-up of the melanophores within the streak into varying numbers of layers, so that there appeared to be more melanophores per unit area of streak than in the non-streaked regions of experimental or in normal animals. The melanophores were randomly oriented within the streak area. The melanophore processes seemed to fuse with one another, but whether they actually anastomosed cannot be stated with certainty. In general, in the regions immediately adjacent to the streak, the long axes of the melanophores were oriented radially about the streak. The striking similarity between this and the radial orientation of melanophores in wound healing as described by Herrick ('32) is discussed below.

In no instance were such streakings visible in the irradiated animals placed in the light, or in the non-irradiated controls in light or darkness (see fig. 1).

The melanophores of the normal animals were randomly distributed, and consisted of a thickened so-called cell body with two or more simple processes. Photorecovery is obviously involved here, for the ability of ultraviolet radiation to induce melanophore streaks is checked if followed by illumination with visible light. Illumination had no effect on the non-irradiated controls, since normal larvae maintained in light and in the dark showed no differences.

There appeared to be a threshold amount of radiation necessary to elicit the streaking phenomenon. In general, this threshold dose was greater in the larger animals, although individual variations were observed.

Repeated doses

Ultraviolet radiation in doses of approximately 2×10^4 ergs per cm^2, amounting to a small fraction of the lethal dose, was administered daily over a period of five weeks to 11 *Rana pipiens* tadpoles ranging in size from 37 mm to 65 mm. One group of animals was kept in light between exposures to ultraviolet light, while the other was placed in the dark. Photorecovery was indicated by findings similar to those obtained in the single dose experiments. Within 3 to 6 days after the first irradiation, animals in the dark exhibited streaks of melanophores dorsally, whereas those kept in the light were not noticeably different from the controls.

Corneal pigmentation

After irradiation with ultraviolet, melanophores appeared at the periphery and, in some cases, toward the center of the corneas of tadpoles of both species if they were kept in darkness. In certain *Rana catesbiana* larvae, a few peripheral corneal melanophores were present normally; in these animals, a greatly increased number of melanophores was produced by irradiation and subsequent maintenance in the dark. No corneal melanophore spotting appeared in animals which were illuminated after exposure to ultraviolet radiation, and in those larvae normally possessing some corneal melanophores, no increase in these could be observed.

Masking experiments

Experiments were conducted to determine whether the production of dorsal melanophore streaks by ultraviolet radiation was a local effect confined to the surface exposed to irradiation, or might be due to the hormonal action of the pituitary gland, since the relationship between the pituitary and the condition of the melanophores in anuran larvae is well established. Swingle ('21) found that engrafting intermediate lobes of the pituitary gland into *Rana catesbiana* tadpoles

induced great expansion of the epidermal melanophores. According to Parker ('48), the pituitary gland-melanophore system in amphibians involves the production of nerve impulses by light impinging on the eyes of the animal. These impulses pass from the retina over the optic tracts and through the deeper parts of the brain to the intermediate lobe of the pituitary. The hormone of this lobe is released and is carried in the blood and lymph to the melanophores of the skin, which thereupon expand.

Since the effect, if any, of ultraviolet radiation on the pituitary gland would presumably be through the eyes, the eyes of 10 *Rana pipiens* tadpoles measuring approximately 40 mm in length were covered with strips of aluminum foil which extended from the posterior border of the eyes anteriorly to the tip of the snout. With the possibility of radiation affecting the pituitary thus ruled out, the larvae were subjected to a single dose of ultraviolet radiation of approximately 2×10^5 ergs per cm^2. The animals were anesthetized in a 1:3000 solution of chloretone during irradiation. Following irradiation the masks were removed, and 5 of the animals were placed in light, 5 in darkness. Those placed in the dark developed dorsal melanophore streaks within 3 days after irradiation, these streaks extending no farther anteriorly than the posterior borders of the masks. In animals irradiated without a mask and placed in the dark, the streaking reached farther anteriorly than in the masked animals. The animals which were placed in the light after irradiation while masked exhibited no melanophore streaks.

A group of 6 *Rana pipiens* tadpoles approximately 44 mm long were exposed to 2×10^5 ergs per cm^2 of ultraviolet radiation while anesthetized with chloretone and masked with aluminum foil from just behind the eyes posteriorly to the tip of the tail, thus exposing the eyes and cephalic region to the ultraviolet radiation. In this case, no melanophore streaks appeared in either the animals placed in the dark or in light following irradiation.

The results of these masking experiments indicate that the action of the ultraviolet radiation producing the dorsal melanophore streaks is a local one in which the pituitary gland is not involved.

DISCUSSION

The migration of epidermal cells to an area of injury has been described by Smith ('31) in goldfish and Herrick ('32) in *Rana clamitans* larvae. Matsuomoto ('18) has observed the migration of epidermal cells in vitro in the cornea of the frog. Herrick described the melanophores as assuming a radial arrangement around the edges of an incision, and suggested that this orientation resulted from the movement of non-pigmented epidermal cells which carried the melanophores to the site of injury. The radial orientation of the melanophores as observed by Herrick corresponds closely with that observed in the irradiated tadpoles kept in the dark, as described in this paper.

The migration of epidermal cells in response to injury may also explain the presence of melanophores in the corneas of the *R. pipiens* larvae. The concentration of melanophores was greatest at the periphery of the cornea, indicating that perhaps there was an active migration of epidermal cells onto the cornea, carrying the non-motile melanophores with them.

The increased concentration of melanin-bearing cells in streaked areas may also have been due in part to a passage of melanin particles from actual melanophores, where they are elaborated, into adjacent non-pigmented epidermal cells. Stearner ('46) reported such a transfer of melanin particles in the skin of urodele amphibians.

It appears, thus, that the streaking observed after ultraviolet radiation is comparable to melanophore reactions after other types of injury. It is possible that the formation of melanin in the melanophores is directly stimulated by the action of ultraviolet, since there appeared to be an increased amount of melanin in many of the melanophores within the streaked areas of irradiated tadpoles kept in the dark. The relatively

large size of many of the melanophores with no processes renders doubtful the possibility that they are in the state of contraction which characterizes the normal melanophore response to darkness. The smaller of these melanophores having no processes may be in a state of contraction similar to that described by Torraca as reported by Laurens ('15), in ultraviolet-irradiated *Triton*. They may also represent fragments of larger melanophores.

SUMMARY

1. *Rana catesbiana* larvae and *Rana pipiens* larvae irradiated with single or repeated doses of ultraviolet radiation and placed in total darkness develop pigmented streaks dorsally and isolated melanophores on the peripheries of the corneas. These effects appear at varying times within a few days after irradiation. The threshold of radiation necessary to induce streaks and corneal pigment spots varies with individual animals.

2. Irradiated animals placed under "visible" light show none of these effects, indicating photorecovery.

3. The pituitary gland is not involved in the streaking process, which appears to be a local response.

4. The orientation and configuration of the melanophores in the streaked areas and adjacent to the streaks suggest that the streak represents an effect of local injury induced by ultraviolet radiation.

Photorecovery would seem, in this instance, to represent the repair of injurious or lethal effects brought about in tissue cells by ultraviolet radiation. This immediate repair results in the suppression of the melanophore changes, etc., which would otherwise occur.

ACKNOWLEDGEMENT

We are indebted to Professor Harold F. Blum for valuable guidance and criticism at all stages of these investigations.

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PENETRATION OF THE DESHEATHED TOAD SCIATIC NERVE BY IONS AND MOLECULES

I. STEADY STATE AND EQUILIBRIUM DISTRIBUTIONS

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INTRODUCTION

The evaluation of the penetrability characteristics of vertebrate nerve fibers to ions and molecules involves special difficulties. Peculiar to vertebrate nerve is the question of the relative importance of the myelin sheaths surrounding the individual axons. Moreover, the small size of the fibers requires the use of large numbers, for example, as found in the nerve trunk; this immediately demands methods to distinguish between the intracellular and extracellular phases, as well as to recognize compartments within each of these. Studies of the kinetics as well as of the final distribution of radioisotopes in intact bullfrog nerve have demonstrated some of the possibilities in this respect (Shanes and Berman, '53); however, in this preparation the peripheral connective tissue sheath, known as the epineurium, largely obscures the properties of the fibers themselves (Shanes and Berman, '53; Shanes, '54b).

Although removal of the epineurium is a relatively simple matter which has been practised by physiologists for several years, it has recently been shown to cause an enlargement of the extracellular spaces in bullfrog nerve which is progressive for several hours (Shanes, '51, '53). This renders the prep-

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aration unsatisfactory for many purposes, including those of this and the subsequent study.

The finding that the desheathed sciatic of the toad, Bufo marinus, remains structurally, chemically and functionally stable for many hours and even days in ordinary frog Ringer's at room temperatures (Shanes, '54a) offers the possibility of distinguishing with quantitative precision, between the properties of the fibers and those of the extracellular spaces. The results to be described give promise of achieving this. In the absence of specific information regarding the conditions governing the maintenance of ion concentrations in the axons, no attempt will be made to distinguish steady states from equilibria.

The present report is concerned with the distribution of sodium and potassium in low and high sodium nerves as measured with a flame spectrophotometer, and with the distribution, after complete equilibration, of the radioisotopes Na^{22}, Cl^{36}, $S^{35}O_4$, and C^{14} labelled sucrose and urea.

Sucrose enters to the smallest extent, which is consistent with a restriction to the extracellular spaces. Urea uptake is the greatest and approximates a uniform distribution in all the nerve water. Na^{22}, Cl^{36} and $S^{35}O_4$ entry is intermediate.

The significance of these findings will be discussed from the standpoint of current knowledge of nerve structure.

METHODS

Geiger and flow counters, with conventional commercial scalers, as described previously (Shanes and Berman, '53), were used to estimate the radioisotopes. The usual precautions were taken to avoid coincidence error and to minimize or correct for absorption during counting. Half milliliter samples were dried on stainless steel planchets for counting purposes using methods which improve the uniformity of deposition.

Analyses for the common stable isotopes of sodium and potassium were carried out with the Beckman No. 10300 flame spectrophotometer, the usual care being taken to mini-

mize background and to correct for sodium-potassium interaction (Shanes, '50, '52).

Nerves were equilibrated overnight (at least 16 hours) at 25°C. in experimental solutions — a period which the present and immediately following reports demonstrate to have been quite adequate to assure completeness of distribution of the ions and molecules studied. For slower moving particles, such as sucrose and SO_4, 24 hours of exposure were used routinely.

Frog Ringer's, in which the desheathed toad nerves show excellent survival (Shanes, '54a), was used throughout except when an elevation of fiber sodium was desired. The Ringer's contains 108 mM NaCl, 1.7 mM KCl, 1.1 mM $CaCl_2$, and a pH 7.2 all sodium Sørensen phosphate buffer osmotically equivalent to 1 mM NaCl, all per liter of solution. The increase in fiber sodium was brought about by 16 hours exposure to unbuffered 0.111 M/l NaCl.

All radioisotope solutions were made up directly from radioactive materials as obtained from the suppliers, neutralized where necessary, and diluted in Ringer's; suitable adjustments were made to assure that the "hot" Ringer's was identical in every possible way with the inactive medium. Activities were kept on the microcurie level, and absolute concentrations were low. Thus, in these experiments sucrose,[2] labelled uniformly with C^{14}, never exceeded 2 mg/ml (1 μC/ml) and hence altered the osmotic strength less than 3%. Sulfate is obtainable carrier-free,[3] therefore its concentration was insignificant even when activities of 10 μC/ml were employed. C^{14} labelled urea [4] was used at 0.2 mg/ml, giving an activity level of about 5 μC/ml. Na^{22} and Cl^{36}, both obtained from Oak Ridge, were employed in activities of 1 and 0.3 μC/ml, respectively. Radiopurity was checked by running absorp-

[2] Supplied by the Nuclear Instrument and Chemical Corp. with a purity estimated at better than 99% and an activity of 0.5 μC/mg.

[3] Supplied by the Oak Ridge National Laboratory. Barium precipitations with added SO_4 carrier in solutions exposed to nerve for 24 hours removed over 99% of the radioactivity.

[4] Supplied by Technical Associates. We are indebted to Dr. E. Titus of the National Heart Institute for providing the radioactive urea.

tion curves. All solutions were examined for toxicity by checking action potentials after at least 20 hours of exposure.

Solutions containing the organic radiocompounds and $S^{35}O_4$ were freshly prepared and contained 8 mg% Merck Penicillin G and 16 mg% Merck Streptomycin SO_4.[5] These antibiotics have no deleterious effect on the nerves, as reflected by the constancy of spike amplitudes and spaces, and prevent a 10% breakdown of the sucrose to the monosaccharides which we find to occur at 25°C. during the over-night exposure of the sciatics to the standard sugar solution.[6] Results with sucrose were rendered more uniform with the antibiotics, although the maximum uptake of activity during equilibration was not changed appreciably.[7]

A single procedure was adopted for determining the radioactivity or sodium and potassium levels of the nerves at the end of equilibration. The individual sciatics were shaken in three or 5 ml of distilled water for 24 hours (with the antibiotics present in the case of sucrose and sulfate) and the extracts analyzed or deposited and dried on planchets for counting. The volume employed depended on nerve size. This water extraction technique was adopted when it was found to be effective for the removal of potassium in intact bullfrog nerve (see tables 1 and 2 and RESULTS). The experimental soak solutions were diluted at least 200-fold for counting purposes.

The epineuria were removed in the usual way (Shanes, '54a, b) and the desheathed preparations mounted on glass rods with tantalum wire as described previously (Shanes and

[5] We are grateful to Dr. P. Weinstein, of the National Microbiological Institute, for recommending this combination of antibiotics.

[6] It is a pleasure to acknowledge our indebtedness to Dr. E. Cotlove of the National Heart Institute for making available the facilities, and to Miss E. M. MacEwen for her technical assistance, in running chromatograms and the delicate chemical test for reducing sugar as described by Somogyi ('52) which enabled us to demonstrate the breakdown of the sucrose in the absence of antibiotics and its prevention in their presence.

[7] This suggests that the breakdown in the absence of antibiotics was to particles still too large to penetrate the fibers.

Berman, '53). It was impracticable to work with branch free segments, since these would have been too short. The proximal region which contains a heavily branched area was excluded, while the peroneal and tibial nerves, which lack branches, were included. The few intermediate small branches were cut short. Following equilibration, the nerves were

TABLE 1

The potassium and sodium removed from intact bullfrog nerves with increasing exposure to distilled water

NERVE	BY 2 HOURS	BY 6 HOURS	BY 24 HOURS	NERVE RESIDUE AT 24 HOURS [1]	TOTAL
			POTASSIUM		
			μM/gm initial wet weight		
1	15.3	26.4	34.7	2.3	37.0
2	18.1	31.2	40.9	1.1	42.0
3	16.1	30.2	39.2	1.6	40.8
4	13.3	27.1	37.5	1.9	39.4
5	18.2	31.0	41.2	1.9	43.1
6	14.1	25.4	33.4	2.4	35.8
Mean ± S.E.	15.9 ± 0.8	28.6 ± 1.0	37.8 ± 1.3	1.9 ± 0.2	39.7 ± 1.2
			SODIUM		
			μM/gm initial wet weight		
1	65.2	65.7	67.1	1.7	68.8
2	56.3	61.6	66.2	1.0	67.1
3	57.3	61.3	65.3	2.0	67.3
4	48.9	53.1	55.8	1.6	57.4
5	55.2	57.4	62.7	1.7	64.4
6	56.7	60.3	66.3	0.9	67.3
Mean ± S.E.	56.6 ± 2.1	59.9 ± 1.7	63.9 ± 1.7	1.5 ± 0.5	65.4 ± 0.9

[1] By analysis of the ashed nerve.

gently blotted on highly absorbent filter paper (Whatman No. 5), several millimeters of tissue at either end discarded, and quickly weighed on a Roller-Smith torsion balance (precision 0.1 mg).

RESULTS

Extraction of ions in distilled water. The wide variety of substances to be studied and the losses of Na^{22} and Cl^{36} ob-

served under certain conditions of incineration made desirable a uniform procedure of extraction without ashing for determination of the amounts of the ions or molecules contained in the nerves. To this end, the emergence of ordinary sodium and potassium into distilled water from intact Rana catesbiana nerves, where the epineurium introduces an additional delay in the exit of these ions, was examined as a function of time. A high measure of precision was achieved by withdrawing small samples at given intervals from the same

TABLE 2

Comparison of the sodium and potassium contents of bullfrog nerves, estimated by analyses or counts of the aqueous extracts after 24 hours exposure to distilled water (direct), with those determined from aqueous extracts of the 16 hour incinerated residues of companion nerves (indirect).

	POTASSIUM		SODIUM[23]		SODIUM[22]	
	Direct [1]	Indirect	Direct [1]	Indirect	Direct [2]	Indirect
	μM/gm		*μM/gm*		*Counts/min. mg*	
	37.0	36.5	68.8	74.4	166.3	180.5
	42.0	43.1	67.1	72.1	159.3	165.1
	40.8	37.6	67.3	73.1	173.4	188.1
	39.4	40.2	57.4	73.8	167.4	167.6
	43.1	40.1	64.4	68.9	170.6	181.7
	35.8	35.0	67.3	74.3	153.2	153.9
Mean ± S.E.	39.7±1.2	38.8±1.2	65.4±0.9	72.8±0.9	165.0±3.1	172.8±4.5

All data referred to the initial wet weight.

[1] Residue in nerve after 24 hours included.

[2] Residue in nerve after 24 hours not included; correction for that found with Na^{23} (table 1) gives a mean value of about 170 counts/min. mg.

extracts; in addition, the final estimates were compared with those obtained with ashed companion nerves (see Shanes, '51). The comparison of ashing and water extraction was also carried out with the radioisotope Na^{22}. The results are summarized in tables 1 and 2.

In table 1 potassium can be seen to emerge more slowly than sodium. By two hours the loss of sodium is almost 90% complete, whereas only 40% of the potassium has escaped. After 24 hours the absolute residues of sodium and potassium are about the same — 5% or less of that initially present.

In table 2 are given the cation contents of paired nerves; the figure for one of each pair was obtained from the direct aqueous extract, that of the companion nerve by analysis of the 4 hour water extract of the ash in "Vycor" crucibles after 16 hours of incineration at 500°C. For potassium and Na^{22} the agreement of the results by the two methods is excellent. In the case of Na^{23} — the normal sodium of the tissues — the ashing procedure gives a value systematically

TABLE 3

Sodium and potassium levels and different spaces of desheathed toad nerves equilibrated in Ringer's and unbuffered NaCl

	K	Na^{23}	Na^{22} SPACE	Cl^{36} SPACE	WATER SPACE
NaCl					
	$\mu M/gm$		%	%	%
	16.3 ± 1.0	86.8 ± 1.2	87.9 ± 1.6	65.4 ± 0.9	83.4 ± 0.7
No.	33	33	16	14	10
RINGER'S					
	38.6 ± 0.8	70.2 ± 0.9	70.4 ± 0.9	62.5 ± 0.8	79.5 ± 0.6
No.	38	38	16	14	27

	SUCROSE SPACE	$S^{35}O_4$ SPACE			UREA SPACE
		24 hours	48 hours	Total	
	%	%	%	%	%
	48.9 ± 1.8	60.8 ± 1.2	61.5 ± 1.4	61.0 ± 0.9	86.3 ± 1.0
No.	16	28	16	44	24

The means are given with their standard errors and the number of nerves involved. All data are relative to the final wet weight of tissue.

higher by about 10%. This is suspected to involve contamination, possibly from the glass of the crucibles. Subsequent experiments with toad nerve show a small discrepancy in the opposite direction between Na^{22} and Na^{23} spaces (table 3). In the light of the general agreement obtainable, the simpler and more generally applicable procedure of direct water extraction for 24 hours has been used throughout the present study of desheathed toad sciatics, in which the absence of the epineurium greatly facilitates diffusion (Shanes, '54a).

Steady state or equilibrium concentrations and "spaces". No special significance can be attached to the absolute radioactivity level attained by nerves after equilibration in "hot" Ringer's since this will be proportional to the activity of the Ringer's. However, if the activity/gm wet weight tissue is divided by the activity/ml Ringer's, a figure is obtained which expresses the "space," i.e., the milliliters of nerve water which would be occupied by the particular substance were its concentration the same as in the equilibrating solution (*cf.* Fenn et al., '34). This is by no means necessarily an actual description of the existing situation, particularly since the thermodynamic activities within tissues are seldom known with certainty. The possibility of "binding" or of altered concentrations resulting from electrical potential differences across membranes and other properties of living tissue must ultimately be considered. Nevertheless, when defined on a purely operational basis, the "space" of a substance is a useful index of uptake and will be used in this sense for the descriptive portion of this report.

In table 3 are compared the absolute sodium and potassium levels as well as different spaces after suitable equilibration of the nerves in Ringer's or NaCl. The data in this table, as well as those to follow, are referred to the final rather than to the initial wet weight. No difference is involved for Ringer's soaked nerves, since these do not change in weight over long periods (Shanes, '54a). NaCl, however, causes a 5% increase in weight overnight; this is seen in table 3 to be accompanied by a small, proportionate increase in chloride space over that found in Ringer's nerves.

The water space was determined from the loss in weight (a) upon drying to constant weight at 100°C. and (b) by drying *in vacuo* at 45°C. or less. Preparations dehydrated first by the second method and then by the first showed no additional weight change.

The water content is 10% smaller than the urea space, a difference which appears to be significant; whether this reflects the tendency of urea to combine with lipid or other components

(Schlenk, '54) cannot be stated at present. In any case, these two spaces are the largest for Ringer's soaked nerves. The sodium space of NaCl treated nerves is of the same magnitude. Such large values are indicative of the presence of these substances in both the fibers and the interstitial region. Sucrose, on the other hand, exhibits the smallest space, which may signify its exclusion from at least one phase in nerve.

The figures for K, Na, Cl, sucrose and water are comparable to those previously reported for frog nerve (Fenn et al., '34, Shanes, '52, Shanes and Berman, '53). The sulfate space equals the chloride space, which appears to be contrary to the finding by Amberson et al. ('38) for mammalian nerve that sulfate does not exchange completely for chloride. However, the penetration of the fibers by sulfate appears to be very slow (Shanes and Berman, '55), so that the limited duration of the earlier experiments may have been inadequate for full exchange. Whether this will account for the smaller radiosulfate space relative to chloride in mammalian muscle (Walser et al., '54) cannot be answered at present. The possibility of species or tissue differences must also be examined.

The identity of the SO_4 space at 24 and 48 hours establishes the completeness of equilibration at the shorter time. Similarly, the space for Na^{22} in low and high sodium nerves agrees closely with that of the inactive ion, demonstrating the adequacy of 16 hours of equilibration for this radioisotope. It should be noted that the Na^{23} figures in table 3 must be divided by the sodium concentration of the Ringer's (108 μM/ml) to give the spaces, which leads to values smaller than for the radioisotope. The difference is not considered real.

The gain in sodium by nerves exposed to NaCl is about equal to the loss of potassium. The figures for Na^{23} and K in these preparations, given in table 3, must be multiplied by 1.05 to correct for the water uptake of the nerves since they are referred to the final weight. When this is done, we obtain 90.1 and 17.1 μM/gm initial wet weight, respectively, hence the sodium increment is 19.9 μM and the potassium loss is

21.5 μM per gram wet weight in NaCl. Since the increment of both Cl^{36} and water under these conditions may reflect the entry of 4 of the 20 μM of sodium with chloride, the sodium-potassium exchange actually may not be so close to 1:1.

The effect of exposure to unbuffered NaCl on spike amplitudes, as well as the reversibility of ionic and spike changes

TABLE 4

The contents of the non-radioactive isotopes of sodium and potassium and the spike amplitudes of paired desheathed toad nerves after (A) 40 hours in Ringer's, (B) 16 hours in NaCl, and (C) 16 hours in NaCl followed by 24 hours in Ringer's.

ANIMAL	SODIUM			POTASSIUM			SPIKE		
	A	B	C	A	B	C	A[1]	B	C
	μM/gm			*μM/gm*			*millivolts*		
1	68.8		75.0	37.8		26.6	22–27	4.5	6.0
2	61.1		80.6	42.4		26.8	26–31	5.0	6.0
3	66.5		79.6	42.1		27.1	23–26	7.0	5.0
4	71.1		82.9	37.4		26.3	26–28	6.5	7.5
5		77.6	74.6		17.9	33.6		9.0	10.0
6		79.5	72.7		18.0	32.5		10.0	10.0
7		76.0	71.8		24.1	38.6		17.0	10.5
8		87.7	83.3		12.8	31.7		5.5	7.5
9	62.1	77.6		45.4	23.1				
10	62.8	77.0		43.9	25.3				
11	72.6	83.8		43.0	18.1				
12	66.1	74.9		40.6	27.3				
13		85.5	84.0		17.5	27.3		2.0	5.0
14		95.2	91.5		8.3	32.6		7.0	14.5
15		85.6	92.0		23.3	26.4		10.5	6.0
Mean	66.4	81.9	80.7	41.6	19.6	30.0	24–28	7.7	8.0
± S.E.	±1.5	±1.5	±2.1	±1.0	±1.7	±1.4		±1.2	±0.8

All values referred to the final wet weight.

[1] The first value is that for freshly desheathed nerve, the second after 40 hours.

upon return to Ringer's, were also examined. The results are summarized in table 4. The spike amplitudes are very much reduced in NaCl and no recovery is evident upon return to Ringer's. Despite the markedly reduced electrophysiological activity, high amplifier amplifications reveal spontaneous repetitive activity in many of the NaCl treated nerves; none was seen in the control preparations. This is suggestive of functional depression rather than death as the basis for the

low spikes. In keeping with this, an intracellular rise in sodium and fall in potassium may be expected to reduce the spike of individual fibers (Hodgkin and Huxley, '52; Hodgkin and Katz, '49). Moreover, a slowing of conduction velocity and the attendant dispersion of spikes from different fibers also will reduce the action potential; such a broadening of the compound spike was quite evident in our preparations. The postulated effects on spike amplitude and velocity in individual fibers attend repetitive activity in the squid giant axon; as in NaCl-treated toad nerve, they are not reversed under *in vitro* resting conditions (Shanes, '54c). It is probable that in many fibers the spikes were depressed to the point of block, but again this may reflect inadequate impulse production rather than actual damage. The extremely small increase of the chloride space in NaCl is consistent with this view. The close identity of the permeability to sodium in Ringer's and NaCl treated preparations, indicated by the kinetic studies to be described in the immediately following communication, is further evidence of the structural integrity of NaCl treated fibers.

Comparison of columns B and C in table 4 demonstrates that following 16 hours exposure to NaCl return of the nerves to Ringer's leads to no ejecion of sodium, while an uptake of potassium, although not complete, nevertheless is substantial. With respect to sodium, therefore, this preparation is in a steady state. The weight has been observed to remain unchanged upon return to Ringer's; whether this reflects a "binding" of potassium, or the absence of an osmotic response to the increase in ionic strength — imposed perhaps by the myelin sheath — cannot be stated. In any case, table 4 provides an example of potassium uptake independent of sodium; the loss of potassium during anoxia can also occur without sodium movement (Shanes, '52).

DISCUSSION

The data which have been described are of significance in three respects, although only the third will be considered in

detail: (1) The usually larger shifts of potassium than of sodium, and in particular the uptake of potassium in the absence of any outward movement of sodium, clearly point to a mechanism capable of potassium transport independent of sodium. As pointed out, the lack of a corresponding movement of water in the latter case is suggestive of potassium "binding."[8] Stone and Shapiro ('48) and Folch ('47) find evidence for a binding of 25–30% of brain potassium. However, further research is required to establish this as a property of living nerve, particularly since the possibility cannot be excluded that an osmotic change was prevented by the myelin sheaths. (2) The demonstration that the sulfate ion remains restricted to the same volume and that sodium and potassium levels are unchanged for 40 to 48 hours exposure of the desheathed toad sciatics to Ringer's adds further evidence for the remarkable stability of these preparations over long periods of time (Shanes, '54a). This is of considerable importance for studies such as the present one since it permits adequate equilibration and thereby assures valid "spaces". It is also important for quantitative analyses of the kinetics of ion and molecule movement which depend on strictly steady state or equilibrium conditions. (3) The different spaces which have been found call attention to the complexity of the factors governing the distribution of substances in nerve.

Up to this point the term "space" has been used purely operationally as an index of relative uptake without assumptions regarding the actual distribution. On the basis of the present and recent data on the histological, chemical and

[8] The term "binding," as employed in the literature, refers to an association of ions with larger particles which may be (a) exceedingly loose, as for strongly dissociated compounds, so that the thermodynamic activity of the ions in solution is essentially that of their concentration, (b) firmer, so that the thermodynamic activity is reduced (i.e., the free ion is removed in part from solution) but can still exchange with itself or with sufficiently similar ions (Klotz, '50), and (c) very tight, with the result that the ion is removed from solution and inexchangeable. Type (b) is evidently the case for chloride binding by connective tissue since the radioisotope completely exchanges with the stable isotope (Manery and Haege, '41); the possibility of such binding of intracellular components renders its detection difficult.

electrical properties of nerve and its components, a balance sheet of electrolyte distribution will now be attempted.

An important question to be answered at the outset is the nature of the phase or phases occupied by sucrose. The simplest view, in the light of the classical evidence for impermeability to this disaccharide (Höber, '45) and of its restricted uptake, is to consider its location to be in the extracellular spaces. From the sucrose space, then, the aqueous content of the extracellular phase is 0.5 ml/gm of desheathed nerve. The remaining water, 0.3 ml/gm, must be in the cells.

The substantial volume occupied by the myelin sheaths — approximately equal to the axons in living amphibian fibers (de Renyi, '29; Schmitt and Bear, '37) — raises the additional question as to whether the cellular water is that in the axoplasm alone, or in both myelin and axon. If the former, then it can be shown that sucrose must penetrate the myelin, which is therefore in chemical equilibrium with the surrounding medium and more properly part of the extracellular spaces.

The two possibilities lead to very different proportions among the three major phases of nerve, which may be estimated in terms of total weights since the densities are about equal.[9] If sucrose is extrafibrillar, and the dry weight of this extracellular phase is the same as for the epineurium, viz., 15% [10], then the interstitial spaces are 0.6, and the axons and myelin each 0.2 of the nerve weight. If, on the other hand, sucrose is excluded only from the axon, which may be considered to have a dry weight of 10% as in the squid giant fiber (Bear and Schmitt, '39), the axons, sheaths and extrafibrillar spaces each constitute one-third of the nerve by weight.

The two-fold difference in the predicted dimensions of the interfibrillar phase should allow selection of the correct

[9] This follows from the relative proportions of lipid, proteins and water in myelin (Schmitt et al., '41) and from the high content of water in the rest of the nerve.

[10] Preliminary determinations on 6 groups of 5–6 sheaths give an average value of 17%; 15% is taken as a round number.

interpretation on the basis of good histological sections. The serious distortions introduced by chemical fixation techniques make the usual sections of questionable validity. Birren ('54), who has compared these with preparations using the freeze-dry method, finds that the latter leads to an extrafibrillar area which is 36% of the total within the perineurium of rat nerve.[11] A much larger interstitial area is unlikely in toad nerve, so that histological evidence favors the view that sucrose is excluded only from the axon.

Other considerations also support this conclusion. The dependence of internodal resistance on the salinity of Ringer's, demonstrated by Maruyama's ingenious measurements on single fibers (Tasaki, '53), would be expected from the penetrability of the myelin to ions.[12] This important finding, as well as the absence of the cellular membrane potential across the myelin sheath (Tasaki, '52), serve to establish the sheath as more or less in equilibrium with the medium and hence as a part of the extracellular space at least with respect to electrolytes.

From this standpoint, the term "extracellular," commonly employed to delineate the region of the nerve in intimate fluid contact with the surrounding medium, is not satisfactory. Consequently, the aqueous volume between fibers should be designated by "interstitial," "interfibrillar," or "connective tissue" (c.t.) space. This will be followed henceforth.

Accepting the sucrose space as a measure of accessible nerve water in the myelin and c.t. space, then, and using

[11] Osmic acid fixation leads to a still smaller interstitial area (Birren, '54).

[12] Maruyama's sheath resistance-Ringer's salinity curve can be shown to be that obtainable for two resistors in parallel, one of which varies proportionally with the resistance of the Ringer's and the other remaining constant until the salinities fall below that of Ringer's; in the latter case the "fixed" resistor declines by 25% when the salinity is 25% of normal. Our calculations indicate that in Ringer's, in which the myelin resistance is 0.16×10^6 ohm cm^2 (Stämpfli, '54), the salinity sensitive resistance measures 0.8×10^6 and the other 0.2×10^6 ohm cm^2. The former may correspond to electrolyte contained in the Schmidt-Lantermann clefts, which now are regarded as normally present in the sheaths of fresh fibers (Fernández-Morán, '52) to an extent which would make the total internode conductivity of the same order as that at the node (Gasser, '52).

the 10 and 15% dry weight estimates for axoplasm and the interfibrillar region, we obtain the electrolyte distribution shown in table 5 for desheathed toad nerves. The equilibrating solution is Ringer's containing 108 μM/ml sodium, 111 μM/ml chloride and the other usual components.[13]

TABLE 5

Estimated distribution of electrolytes and water in 1 gm wet weight of Ringer's equilibrated desheathed toad nerve

REGION	AQUEOUS VOLUME	TOTAL WEIGHT	POTASSIUM CONTENT	SODIUM CONTENT	CHLORIDE CONTENT
	ml	*gm*	*μM*	*μM*	*μM*
Axon	0.3	0.33	37.8 [1]	19 [1]	14 [1]
Myelin	0.2 [2]	0.33	0.3 [3]	22 [3]	22 [3]
C.t. space	0.3	0.34	0.5 [3]	32 [3]	33 [3]

[1] By subtracting myelin and c.t. space estimates from the total. Kinetics studies indicate that the sodium and chloride figures are too large (Shanes and Berman, '55).

[2] By subtracting axon and c.t. space estimates from the total nerve water.

[3] On the assumption the contained water is Ringer's.

One outcome of these calculations is that the myelin is 60% water. This is not inconsistent with available literature. The earlier work of Schmitt and his associates (e.g., Schmitt et al., '41) demonstrated that the sheaths of living fibers consist of mixed lipids alternating with protein-water layers. Their measurements indicate that water associated with lipid constitutes 30%; this is a minimum figure since that in the protein layers must be taken into account. The basic pe-

[13] Except for the unlikelihood of the small axonal volume to which it leads, the possibility of sucrose exclusion from the myelin sheaths is still consistent with the penetration of the internode by ions. On this basis the following balance sheet is an alternative possibility:

REGION	AQUEOUS VOLUME	TOTAL WEIGHT	SODIUM CONTENT	CHLORIDE CONTENT
	ml	*gm*	*μM*	*μM*
Axon	0.18	0.2	7	1
Myelin	0.10	0.2	11	11
C.t. space	0.51	0.6	55	57

In contrast to the situation in table 5, the axonal content of chloride is negligible. This is contrary to the finding with kinetics data (Shanes and Berman, '55), and therefore supplements the histological indications of a different distribution.

riodicity, including lipid and protein layers, is about 170 Å. Recent electron microscope observations of frozen dried sections (Fernández-Morán, '52), in which the distortions of preparation are minimal, reveal lamellae which occupy only about half of the basic period, hence a large part of the remainder, viz., about 50%, may therefore contain water. While the methods of x-ray microradiography require careful evaluation (Brattgård et al., '53), it is nevertheless of interest that this technique has indicated a myelin water content of 60–70% (Engström and Lüthy, '50).

How much of this water is accessible to the electrolytes of the medium, and the extent to which the proportions of cations to anions is modified by the components of the myelin, deserves further study. The close identity of the urea and water spaces suggests that all nerve water removed by drying, and presumably all within the sheath, is available to the ions. However, the possibility of urea binding (Schlenk, '54) makes the use of additional criteria desirable.

The postulate of a large Ringer's content for the myelin sheath, which has a high radial diffusion or electrical resistance, is analogous to the situation already described for the epineurium (Shanes, '54a, b). In both instances most of the electrolyte is considered to reside between concentric layers — the lipid lamellae in the case of the myelin — which are the source of resistance. The presence of layers of high electrical conductance between myelin lamellae is consistent with the low capacitance of the sheath (Tasaki, '53; Stämpfli, '54) since the lamellae would act as capacitors in series.

The figures for sodium and chloride in the axon are larger than indicated by the kinetics of exchange (Shanes and Berman, '55). This may not be due to analytical variability alone, for the binding of chloride by connective tissue (Manery et al., '38) and of sodium by lipids (Christensen and Hastings, '40; Folch, '47) could contribute to this difference.

For example, if the 15% estimate of dry weight for the interfibrillar region represents connective tissue, it constitutes 50 mg/gm nerve. From the data given by Manery et al.

('38), corrected as they recommend for the cellular water, one obtains 50 μM of excess chloride per gram of dried mammalian tendon. Applying this figure to toad nerve, we obtain 3 μM as the amount of chloride that may be bound. Actually, twice this figure is probably absent from the axon according to the measurement based on the kinetics of chloride self-diffusion.

A rough estimate of the sodium binding which would occur, if the cephalin of myelin is comparable to that in mammalian nerve and has the binding capacity of extracted cerebral cephalin, shows it to be far in excess of that to be expected from available data. Thus, the difference in the dry weights and cephalin contents of unmyelinated and myelinated nerves, from the most complete data given by Brante ('49) in his table 8 (splenic of the cow and sciatic of the rabbit), may be taken as the contributions by the myelin of the sciatic. From this, about half of the dry weight is myelin and of this 30% is cephalin. A mole of extracted brain cephalin binds 0.6 equivalent of base at pH 7 (Christensen and Hastings, '40). Since the molecular weight of brain cephalin is about 1000 (Brante, '49), the 0.13 gm of dry myelin in table 5 would correspond to 0.04 gm or 40 μM of cephalin, which should be able to bind 24 μM of sodium. This would leave none for the axoplasm. In view of the strong evidence presented in the immediately following communication for sodium in the axon, we propose that the cephalin binding capacity in myelin is substantially less than that obtained with extracts.

Concluding remarks. While some refinement of the values in table 5 appears possible from the kinetics of ion exchange to be discussed in the following paper, nevertheless the uncertainties inherent in the approximations which have been necessary point up the need of the following for a more precise analysis:

1. A comparison of vertebrate unmyelinated and myelinated nerves, preferably from the same animal, with respect to protein and lipid content as well as to spaces. This would

more clearly delineate the properties of myelin as well as that of the other components.

2. A study of cross-sections prepared by the freeze-dry method, also on the same preparation, for an improved estimate of the relative volumes of axons and myelin as well as a check on the connective tissue space. The calculations of table 5 have been restricted to data from living fibers, but the pertinent microscopic information is available only for frog fibers.

3. An examination of the electrolyte binding characteristics of toad connective tissue. The epineurium, so easily obtained from the sciatic nerve, offers a sample of such tissue for study, although satisfactory techniques for handling it must be developed.

4. Corroboration of the assumption of negligible base binding by the cephalin within the myelin. The absence of a pH effect on the sodium content might provide such confirmation, since at pH 4 cephalin binds between one-half to one-third as much base as at pH 7 (Christensen and Hastings, '40).

SUMMARY AND CONCLUSIONS

Sodium and potassium are removable by exposure of nerves to distilled water for 24 hours. This extraction procedure has been adopted to determine the extent of uptake of C^{14} labelled urea and sucrose and of $S^{35}O_4$, Na^{22}, and Cl^{36} by desheathed toad nerves after adequate equilibration in Ringer's containing these radioisotopes. The urea and water spaces are the largest, the former being slightly but significantly larger than the latter. Sucrose is taken up to the smallest extent. Sodium, chloride and sulfate occupy intermediate spaces. In NaCl treated nerves the space for sodium increases by potassium-sodium exchange to about that for water, while that for chloride undergoes only a slight increase proportional to the gain in weight. Spikes are very much depressed after 16 hours of exposure to NaCl solutions, but a low level of repetitive activity frequently remains. Return to Ringer's

for 24 hours, after 16 hours of exposure to NaCl, leads to a substantial uptake of potassium without rejection of the excess sodium and without a change in weight; spike amplitudes remain depressed.

The data which have been accumulated demonstrate (a) chemical and functional stability of desheathed toad nerves kept in Ringer's for at least 40 hours, (b) a high sodium preparation is obtainable by 16 hours exposure to unbuffered NaCl which is still intact according to several criteria and which remains in a steady state with respect to sodium upon return to Ringer's and (c) an uptake of potassium which can be independent of sodium movement. On the basis of these and other available facts the suggestion is made that the water of the myelin sheath as well as of the interfibrillar region is in equilibrium with the surrounding medium. From this standpoint, a tentative balance sheet of ion distribution is presented for desheathed toad nerve.

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PENETRATION OF THE DESHEATHED TOAD SCIATIC NERVE BY IONS AND MOLECULES

II. KINETICS

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FIVE FIGURES

INTRODUCTION

As already pointed out (Shanes and Berman, '53), the loss of radioisotopes from nerve, after a suitable equilibration period, to frequently replaced, non-radioactive solution permits a very accurate reconstruction of the "desaturation curve," i.e., the time course of decline of tissue radioactivity. The remarkable stability of desheathed toad nerves under suitably controlled conditions permits the kinetics to be followed under strictly steady state or equilibrium conditions (Shanes, '54a; Shanes and Berman, '55). The studies to be described are restricted to these conditions.

For an interpretation in multifibered preparations of desaturation curves for ions like sodium and chloride, which may be present in the axons, data are also required on the emergence of substances which do not enter this part of the fiber. Sucrose has been selected for this purpose, since its small uptake indicates that it is excluded from the axoplasm (Shanes and Berman, '55).

The time course of escape of radioisotopes will be shown to involve at least two, and possibly three, stages. An initial rapid one is largely, but not completely, described by homo-

geneous cylinder diffusion theory. A final slow one is exponential in nature. The relation of these components to nerve structure will also be examined. The tentative conclusion is reached that nodes and internodes contribute to ionic transfer.

METHODS

The techniques for following the emergence of radioisotopes into frequently replaced, inactive Ringer's and for extracting the activity remaining in the nerves with water at the termination of experiments, have been described (Shanes and Berman, '53 and '55). The same conventional counting methods were employed. Activity levels were exactly double those used in measuring "spaces."

The initial rapid emergence of radioactivity in desheathed preparations, particularly with sodium and chloride, required the first interval of exposure to non-radioactive Ringer's to be short. Two and one-half minutes at 25°C. and 5 minutes at 4°C. were regarded as the smallest periods consistent with the speed with which the solution could be changed. Removal of solution required but 1–2 seconds; addition of fresh Ringer's took about 20 seconds, which was considered part of the subsequent exposure period. Intervals were increased consecutively by multiples of two. Except for the first collection, activity levels attained by the successive collected samples were of the same order. The error from contamination of succeeding solutions by the residue left from the preceding collection therefore was negligible for most of the samples. The residual volume was probably less than 0.1 ml compared to the 2 ml used for collections, or less than 5%. No correction was attempted for this volume since the total initial radioactivity of the nerves, calculated from the sum of the amounts lost to successive surrounding solutions, including that in the nerve at the end of the experiment, gave spaces identical with those estimated directly, where no volume error is involved (Shanes and Berman, '55). This is demonstrated in table 1. Since the activity loss during each

collection period in the current experiments is calculated from the product of the volume of the collected sample and the activity per milliliter, the neglect of an appreciable residual volume would have given significantly smaller "spaces." The agreement also serves to demonstrate that the various procedures employed, including those involved in the transfer of the nerves from the "hot" solutions to the wash units

TABLE 1

Comparison of the spaces estimated directly (A)[1] and less directly (B) as the sum of the activity from successive collections during emission experiments. Standard errors and the numbers of nerves providing the data for the averages are given.

	SPACES IN NaCl NERVE (%)[2]			
	Na^{22}		Cl^{36}	
	A	B	A	B
	87.9 ± 1.6	84.2 ± 0.8	65.4 ± 0.9	62.3 ± 1.7
No.	16	34	14	8

	SPACES IN RINGER'S NERVE (%)[3]			
	Na^{22}		Cl^{36}	
	A	B	A	B
	70.4 ± 0.9	70.5 ± 1.0	62.5 ± 0.8	63.9 ± 1.3
No.	16	19	14	8
	SUCROSE		$S^{35}O_4$	
	A	B	A	B
	48.9 ± 1.8	54.1 ± 1.7	61.0 ± 0.9	59.3 ± 1.3
No.	16	12	44	11

[1] From Shanes and Berman ('55).
[2] Equilibrated previously in NaCl containing the radioisotope.
[3] Equilibrated previously in Ringer's containing the radioisotope.

(Shanes and Berman, '53), introduced no systematic errors.

All measurements are based on duplicate samples. The maximum range of variability of duplicates was about 5% for Na^{22} and Cl^{36}, and 10% for C^{14} and S^{35}, so that the corresponding standard deviations of the determinations were of the order of 2.5 and 5%. The reconstruction of the desaturation curve for the sum of successive losses, each measured with the same precision, was therefore as accurate for

small movements of radioisotopes, such as occurred at late times, as for the earlier rapid shifts.

Experiments were carried out in constant temperature rooms at 25 ± 1 or 4 ± 1°C., as indicated. The preceding equilibration in both instances was at 25°C., but for low temperature runs the nerves in their equilibration units were first exposed to —8°C. until the desired temperature was reached; all other equipment, solutions and gases were subjected to the experimental temperature for at least 16 hours.

Nerve diameters were checked at 3 mm intervals. In general, the segments employed did not deviate more than 0.1 mm in diameter. The region proximal to the peroneal and tibial branches, up to but not including an area heavy with branches near the major bifurcation, was about 1 to 1.5 cm long and provided the desired uniformity; the few fine intermediate branches were cut off close to the trunk.

EXPERIMENTAL RESULTS

Sodium and chloride

The desaturation curves are given in figures 1 and 2. They describe the time course of exchange of Na^{22} for Na^{23} and of Cl^{36} for Cl^{35}; in all experiments the preparations are in a steady state with respect to Na and Cl (Shanes and Berman, '55). An outstanding feature of these curves is the two distinct components, a major rapid one and a smaller, slow one.

Slow component. The linearity of the slow component when plotted semi-logarithmically demonstrates its exponential nature. This is evident in the curves at 25°C. The limiting slopes at 4°C. probably correspond to the same exponential process as modified by temperature. The slow emergence may therefore be described by two parameters, viz., the slope, which is inversely related to the time constant of this process, and the intercept, obtained by extrapolation of the curve back to zero time. These two parameters will be designated by S and F, respectively.

An exact interpretation of these parameters depends on a complete analysis of the complex geometrical factors which may govern ionic exchange, aspects of which will be discussed later. Nevertheless they are useful when regarded as first

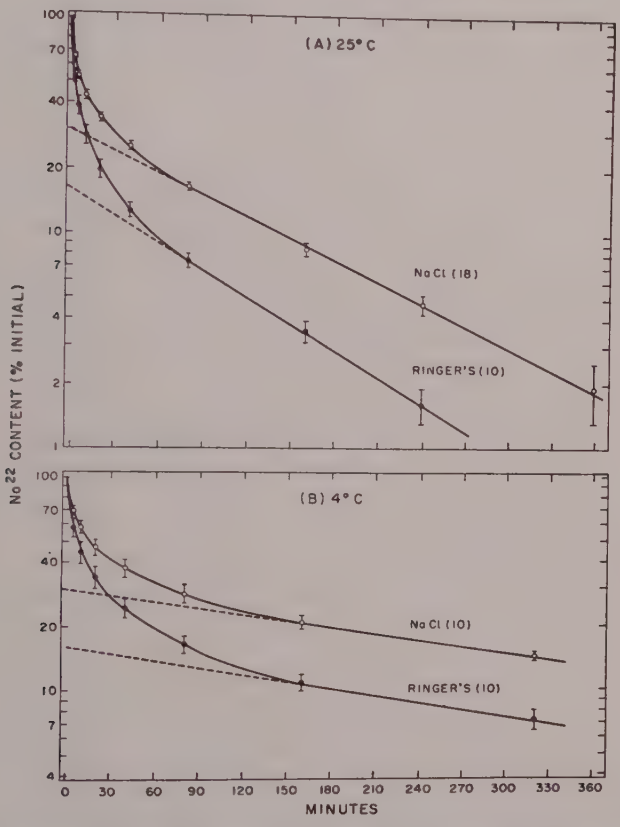

Fig. 1 Decline of the Na^{22} content of NaCl and Ringer's equilibrated nerves by exchange with Na^{23} in Ringer's at 25° and 4°C. In this and subsequent figures the variability of the nerves is shown by the vertical line at each point expressing ± the standard error. Numbers in parentheses give the experimental runs and hence the nerves contributing to each point.

approximations of properties of the fibers themselves, S as a measure of outwardly directed fiber permeability (or, more properly, of ion exchangeability) and F as a measure of the fraction of total radioactivity initially contained in the fibers.

It should be noted that the precision of determination of the small slopes, for the other experimental agents as well as for the monovalent cations, is high because the *difference* between successive points is measured. The standard error

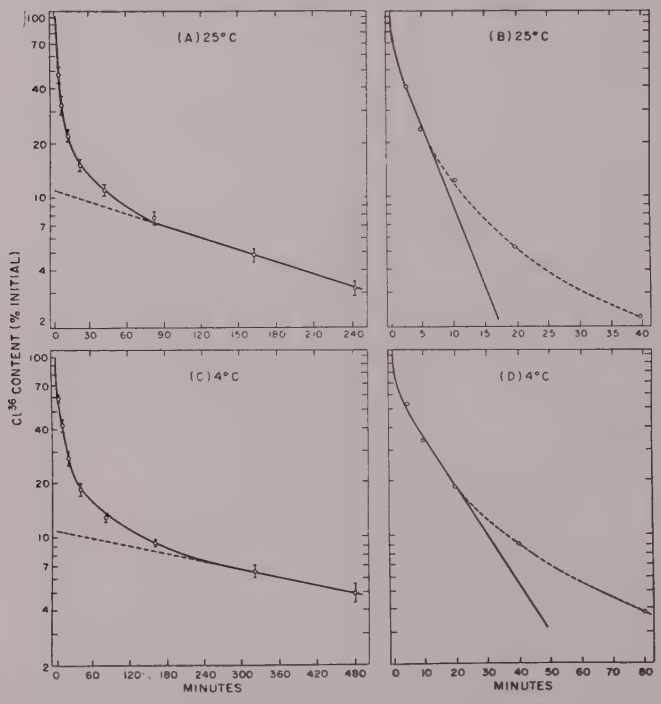

Fig. 2 (A, C). Decline of the Cl^{36} content of desheathed sciatics by exchange with Cl^{35} in Ringer's at 25° and 4°C. In the absence of a significant difference between NaCl and Ringer's equilibrated nerves, the data from both types of preparations have been averaged together. Each point in both curves is the average of data from eight nerves. (B, D) Corresponding corrected curves (dashed lines and circles), obtained by deducting the extrapolated values of the slow component shown in A and C, compared with the theoretical curve for a homogeneous cylinder derived for an average exchange (i.e., self-diffusion) coefficient estimated from the first three corrected experimental points (table 2).

shown on either side of each point in the figures gives the variability among the nerves for that value; but for a given nerve the points at earlier and later times vary in the same direction so that the slope parallels that of the average curve.

The significance of the differences and the slopes is therefore substantially greater than if successive points were completely independent.

As may be seen qualitatively in figures 1 and 2, and quantitatively in table 2, lowering the temperature by 21°C. does not affect F, but decreases S by a factor of about 3.5-fold. This is approximately double the temperature coefficient of

TABLE 2

Parameters describing the emergence of radioisotopes from desheathed toad nerve by exchange (i.e., self-diffusion) or diffusion, calculated for the fast and slow components

PROCESS	PRETREATMENT	TEMP.	SLOW COMPONENT			FAST COMPONENT				
			S	F	$\bar{r}$	k_1	k_2	k_3	$\bar{k}$	D
		°C.	10^3/min.	%	mm	$10^5 \times cm^2/min.$				$10^4 \times cm^2/min.$
	Ringer's	25	9.7	16	0.42	5.6	6.2	4.6	5.5	7.4
Na^{22}-Na^{22}	Ringer's	4	2.5	16	0.43	2.3	2.4	1.9	2.2	4.2
Exchange	NaCl	25	7.7	30	0.42	4.1	4.6	3.9	4.2	7.4
	NaCl	4	2.4	30	0.46	2.0	2.1	2.0	2.0	4.2
Cl^{36}-Cl^{35}	Ringer's	25	5.3	11	0.46	8.1	8.3	6.6	7.7	11.1
Exchange	or NaCl	4	1.6	11	0.50	2.6	3.2	3.0	2.9	6.3
$S^{35}O_4$ Diffusion	Ringer's	25	0.73	10	0.43	1.9	2.0	1.9	1.9	6.2
	Ringer's	25	Neglected		0.41	0.86	0.92	0.89	0.89	3.1
Sucrose	Ringer's	4	Neglected		0.41	0.56	0.56	0.48	0.53	1.6
Diffusion	Ringer's	25	1.8	11.5	0.41	1.1	1.3	1.2	1.2	3.1
	Ringer's	4	0.72	10.5	0.41	0.74	0.79	0.72	0.75	1.6

S = slope of the slow component of the desaturation curves = 1/(time constant).

F = fraction of the total particles giving rise to the slow component, determined by extrapolation to the ordinate at zero time.

$\bar{r}$ = mean nerve radius.

$k_{1,2,3}$ = diffusion or exchange coefficient estimated from the first, second and third points of the curves of the experimental desaturation curves of the monovalent ions and from corresponding points, percentagewise, for sulfate and sucrose.

$\bar{k}$ = mean of k_1, k_2 and k_3.

D = diffusion or "self-diffusion" (i.e., exchange) coefficients in free aqueous solution (see Shanes and Berman, '53).

free diffusion. Another important difference between these observations and those to be expected from free diffusion is in the relative magnitudes of S for chloride and sodium (S_{Cl} and S_{Na}). Thus, the self-diffusion coefficients, *D*, given in table 2, demonstrate that the chloride ion is substantially more mobile than the sodium ion in water, yet S_{Cl} is smaller than S_{Na}, indicating that the factors governing Na^{22}-Na^{23} exchange at late times favor this process more than Cl^{36}-Cl^{35} exchange.

Results of importance from the standpoint of the significance of F and S are the effects on these parameters of treatment of the nerves with NaCl. Attention already has been called to the uptake of sodium which occurs with a loss of potassium in NaCl, and the constancy of nerve sodium thereafter upon return to Ringer's (Shanes and Berman, '55). The Na^{23}-Na^{22} exchange which proceeds after an initial uptake of Na^{22} in these preparations, described by figure 1, therefore occurs with sodium in a steady state. This figure, and the corresponding data for F and S in table 1, demonstrate (a) that F is increased by the increase in nerve sodium and (b) that S of NaCl treated nerves, at a given temperature, is about the same as for Ringer's preparations.

In the light of evidence from unmyelinated systems (e.g., Steinbach, '51; Keynes, '51) that protoplasmic loss of potassium is accompanied by a gain in sodium, it is probable that the elevated sodium in the NaCl treated toad sciatics likewise resides in the axoplasm. Therefore, the rise in F without a change in S indicates (a) that the outward penetrability characteristics of the fibers are little changed by pretreatment with NaCl, (b) that the small, slow exponential component of Ringer's equilibrated nerve is also derived from an intrafibrillar fraction of sodium, and (c) that the rate of outflux of sodium is proportional to the intracellular sodium concentration. Moreover, comparison of the change in F with the rise in sodium provides evidence that it is a direct measure

of the sodium content of the fibers.[1] And since the increased outflux associated with the elevated axonal sodium does not lower the sodium level (Shanes and Berman, '55), the influx must also be proportionately greater, from which it follows that the *inward* penetrability has been permanently increased by pretreatment with NaCl.

No significant difference was seen in Cl^{36}-Cl^{35} exchange in nerves prepared with NaCl or Ringer's. This provides further support for the conclusion (Shanes and Berman, '55) that the small uptake of chloride in NaCl does not occur in intact axons. The data from both types of preparations were pooled for the curves in figure 2.

Fast component. The initial, relatively rapid exchange of the radioisotopes may be expected to represent the process

[1] This may be done as follows. Suppose F is not an exact measure of Na_i (i.e., the intrafibrillar sodium content). Then there is a correction factor, *B*, which will be the same for Ringer's and NaCl soaked nerves (as shown from the analysis of the effect of the diffusion resistance of the interstitial spaces; see p. 221) and which gives the equalities:

$$BF^{R} = Na_i{}^{R}/Na_T{}^{R} \qquad (a)$$

$$BF^{Na} = Na_i{}^{Na}/Na_T{}^{Na}, \qquad (b)$$

where the superscripts *R* and *Na* refer to preparations equilibrated in Ringer's or NaCl and the subscript *T* to their total ion contents. From the space data for Ringer's and NaCl treated nerves (table 3 in Shanes and Berman, '55), the increment of sodium as a result of NaCl exposure is 20 μM per gram of Ringer's nerve. But of this 4 μM must be attributed to an increase in the extracellular spaces shown by the uptake of Cl and water, hence 16 μM is the actual rise in the sodium content of the fibers treated with NaCl.

Now equation b may also be written as

$$BF^{Na} = (Na_i{}^{R} + \Delta Na)/Na_T{}^{Na} = (BF^{R}\,Na_T{}^{R} + \Delta Na)/Na_T{}^{Na} \qquad (c)$$

or

$$B = \Delta Na/(F^{Na}Na_T{}^{Na} - F^{R}Na_T{}^{R}), \qquad (d)$$

in which ΔNa is the increment in sodium. From table 2, F^{Na} is 0.3 and F^{R} is 0.16 and, from the sodium space data of table 3 from Shanes and Berman ('55), calculation gives $Na_T{}^{Na} = 91\ \mu M$ and $Na_T{}^{R} = 73\ \mu M$. Hence

$$B = 16/(27.3 - 11.7) = 1.03$$

Within the precision limits of our estimate of ΔNa, then, *B* is equal to 1. In other words, *F*, the zero time intercept of the slow component, is an exact measure of the fraction of the nerve radioactivity which is in the axon. Therefore, of the 73 μM/gm sodium in Ringer's soaked nerve, 12 resides in the axoplasm. Similarly, of the 69 μM/gm chloride in the same preparation, 7.6 may be in the protoplasm. These figures are almost half of those obtained by simply deducting myelin and connective tissue estimates from the totals (Shanes and Berman, '55).

occurring in the extra-axonal spaces. A suitable procedure is required to quantitatively express the exchangeability of ions in this region of the nerve. If the thin perineurium, several micra thick, remaining on the desheathed toad sciatic (Shanes, '53, '54a) were the limiting barrier, then the emergence would be strictly exponential and the exchange would be conveniently expressible as a time constant. However, such is not the case for the curves shown in figures 1 and 2. This remains true if an attempt is made to correct for the possible contribution of the slow component at early times by subtracting the values obtained by extrapolation of the "tail" to zero time, as in figures 2 B, D and 3.

An alternative approach is to apply the derivation for a homogeneous cylinder (Hill, '28), the applicability of which may be tested by the constancy of the effective diffusion or self-diffusion (or exchange) coefficient that can be calculated for each time interval (see Shanes and Berman, '53). Before doing so, however, the question must be answered as to whether the contribution by the slow component, estimated by extrapolation to zero time, should be deducted first. The necessity for this correction is indicated by the decrease in the initial percentage loss per unit time which would be obtained if, as in NaCl treated nerves, the slow component were derived from a much more substantial fraction of the total radioactivity; uncorrected, this would lead to a smaller estimated diffusion coefficient without any actual change in the diffusion characteristics of the extracellular spaces. Granted the need for the correction, then, the question remains whether the slow component is correctly represented by a simple extrapolation of the linear semi-log plot to zero time. That such is actually the case for sodium has been verified by demonstrating that the increment of F by NaCl treated fibers coincides with the additional uptake of ions.[2] For uniformity, the correction will also be applied to chloride. Justification for this is seen subsequently in the internal consistency of the calculations.

[2] See footnote 1, page 207.

In table 2 are given the results of the computations from cylinder theory for the first three experimental points, and in figures 2 B, D and 3 the corresponding theoretical curves are compared with the experimental ones. In general, the

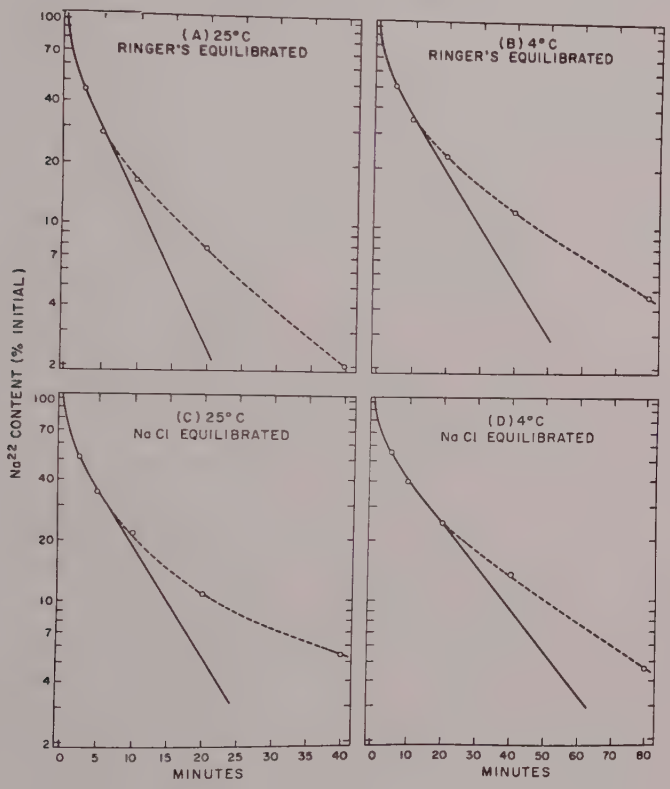

Fig. 3 Decline by exchange at 25° and 4°C. of the Na^{22} content of NaCl and Ringer's equilibrated nerves (broken line and circles) after correction of the curves in figure 1 as in figure 2B, D. These are compared with curves for a homogeneous cylinder (solid lines).

coefficients for the second interval (k_2) tend to be slightly larger than the first (k_1), and those for the third (k_3) to approach the first again. This may be related to technical procedures as pointed out in METHODS. Thus, the activity of the solution collected at the end of the first interval was considerably greater than in subsequent ones. Consequently

a small amount of fluid remaining in the perfusion units might add appreciably to the activity of the second collection, which would make the second value of k greater than the first. Because over 75% of the interstitial activity was removed by the third collection, as may be seen in figures 2 B, D and 3, the mean coefficient, $\bar{k}$, obtained from these three collections, is regarded as representative of the radial diffusion process. The possible significance of the deviation at late times will be discussed shortly in conjunction with the sucrose data.

The values obtained are compared in table 2 with those for corresponding aqueous solutions. It is apparent that the free diffusion process is approximately 20 times faster than in desheathed toad nerve. The *relative* exchangeability of sodium and chloride is the same as for free diffusion, which suggests that steric factors, for example by increasing the mean path length and decreasing the effective diffusion area, are largely responsible for the lower coefficients. The temperature coefficients, however, run systematically higher for the nerves, averaging 2.4 for a change of 21°C., compared to about 1.8 for free aqueous diffusion; from this, at least part of the movement of these ions appears to involve a phase with a greater temperature sensitivity than water.

Thus, we emerge with the view that in desheathed toad nerve the radial outflux of sodium or chloride ions under steady state or equilibrium conditions is attributable to at least two phases, an "extracellular" one with an effective self-diffusion coefficient one-twentieth that of free diffusion and with a somewhat greater temperature coefficient, and an intracellular one with a still greater resistance to exchange and a greater sensitivity to temperature.

Sulfate

In figure 4 A two distinct stages of $S^{35}O_4$ emergence are evident as for Na^{22} and Cl^{36}. Correction for the limiting slope taken as the slow component (fig. 4 B) gives a fast stage which fits homogeneous cylinder theory for over 90%

of the curve. F is about the same as for chloride and S about 1/7 that for the monovalent anion (table 2). While the equality of F_{Cl} and F_{SO_4} is consistent with the equality of the chloride and sulfate spaces, it is not in keeping with concepts of simple membrane equilibria. For example, if only membrane potential governed the distribution of these ions, we should find $[(Cl)_i/(Cl)_o]^2 = (SO_4)_i/(SO_4)_o$.

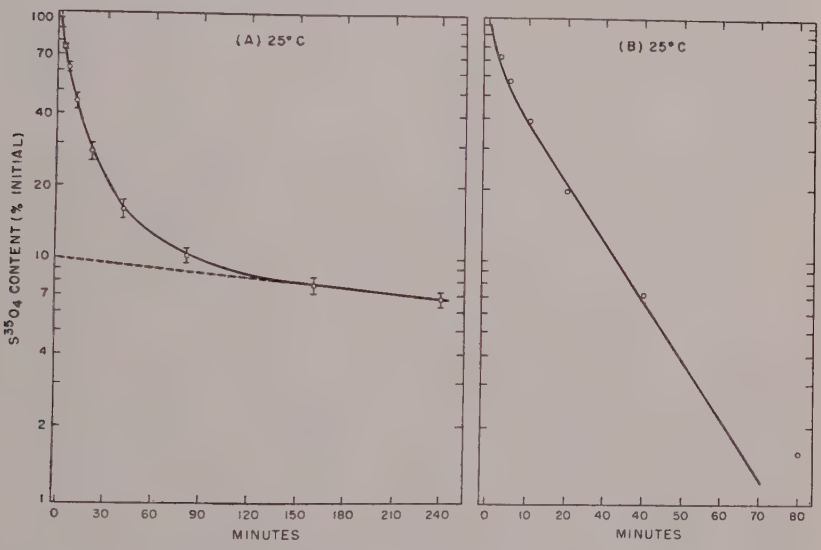

Fig. 4 (A) Decline of the $S^{35}O_4$ content of desheathed sciatics by diffusion into Ringer's. Each experimental point is the average of data from eleven nerves. (B) Circles are the experimental points obtained after correction for the slow component extrapolated as shown in A; the solid line is the theoretical curve for a homogeneous cylinder based on the k values in table 2.

Taking the axon volume indicated by the spaces (Shanes and Berman, '55), we would expect F_{SO_4} to be almost 1/4 of F_{Cl}. S_{SO_4} represents a time constant of 23 hours — about the equilibration time — so that the beginning of the experimental run axonal uptake may have been only 63% complete. On this basis F_{SO_4} would be 16% at equilibrium, or even greater than F_{Cl}. Although no significant difference was seen in the sulfate space after 24 and 48 hours (Shanes and Berman,

'55), the increment in *total* space, to be expected over an additional interval equal to the time constant, is less than 4%, which may well have been too small for detection.[3]

The very slow penetration suggested by these data provides an explanation for the incomplete exchange of sulfate and chloride observed by Amberson et al. ('38) in their *in vivo* experiments of limited duration. A similar explanation may underlie the small radiosulfate space of mammalian muscle relative to chloride (Walser et al., '54), although the possibility of species or tissue differences also requires examination.

Another interesting problem arises when the effective extracellular diffusion coefficient for sulfate, k_{SO_4}, is compared with k_{Cl} and the corresponding values for free diffusion. $SO_4^=$ is present in such low concentration, and other electrolytes in such high concentration, that D_{SO_4} may be taken as that for the individual ion (Arrhenius, 1892; Abegg and Bose, 1899); this value is slightly more than half of D_{Cl} (table 2). But k_{SO_4}/k_{Cl} is 0.25, so that unlike the relative values of k for sodium and chloride, that for sulfate indicates that the divalent ion encounters a greater interference in the extracellular spaces than either of the monovalent ions. This is also true for sucrose.

Sucrose

Although diffusion theory for a homogeneous cylinder appears to account satisfactorily for over 75% of the ionic exchange presumed to occur outside the axons, we have seen that it by no means provides a complete description of this process. This is apparent from the progressively decreasing values of the effective self-diffusion coefficient, k, when esti-

[3] Over 99.5% of the radioactivity of $S^{35}O_4$ containing reservoir solutions was removed by barium precipitation after addition of carrier. Similarly, at least 95% of the activity left in the nerves at the end of the experiments shown in figure 5 was removable; chemical tests gave no evidence that S^{35} in the nerve residue was in a form other than free sulfate.

mated for late times, which is reflected by the continuous decrease of the slope of corrected experimental desaturation curves when plotted semi-logarithmically; simple diffusion theory gives practically a constant slope at late times on the same coordinates (figs. 2 B, D, 3, and 4 B).

In general, the divergence from theory is small when considered in terms of the absolute per cent involved; on semi-logarithmic coordinates the discrepancy is exaggerated. Nevertheless, the precision with which later points are measured, as well as the consistency of the divergence, appear to rule out experimental error as the cause.

The possibility remains to be explored that the apparently smaller exchange coefficients obtained at late times reflect a real property of the extra-axonal region. For this purpose the diffusion at low concentrations of an agent such as sucrose, which may be restricted to this region (Shanes and Berman, '55), was examined.

The desaturation curves for C^{14} labelled sucrose are shown in figure 5, where they are also compared with corresponding theoretical curves for diffusion from a homogeneous cylinder. In table 2 are given the effective diffusion coefficients estimated directly (i.e., without any attempt at correction for a slow component) from the experimental points corresponding to the same degree of desaturation as in the sodium and chloride computations; the theoretical curves in figure 5 A, C were calculated from these coefficients.

Comparison of the curves in figures 2 B, D and 3 with those in figure 5 A, C reveals a general similarity between the fast component of sodium and chloride emergence and the diffusion of sucrose. For example, in each instance 80% of the radioisotopes is described by the theoretical relations for a homogeneous cylinder. Moreover, the experimental data deviate at late times so as to suggest smaller diffusion coefficients for a small part of the diffusion process. In the case of sucrose the possibility of breakdown or other modification of the molecule was eliminated as a factor by chemical test

(Shanes and Berman, '55).[4] The possibility that the slight rise in activity of the initially inactive Ringer's bathing the nerves delays the emergence of the radioisotope also may be rejected (see Shanes and Berman, '53). In keeping with the findings with sodium and chloride, therefore, the conclusion

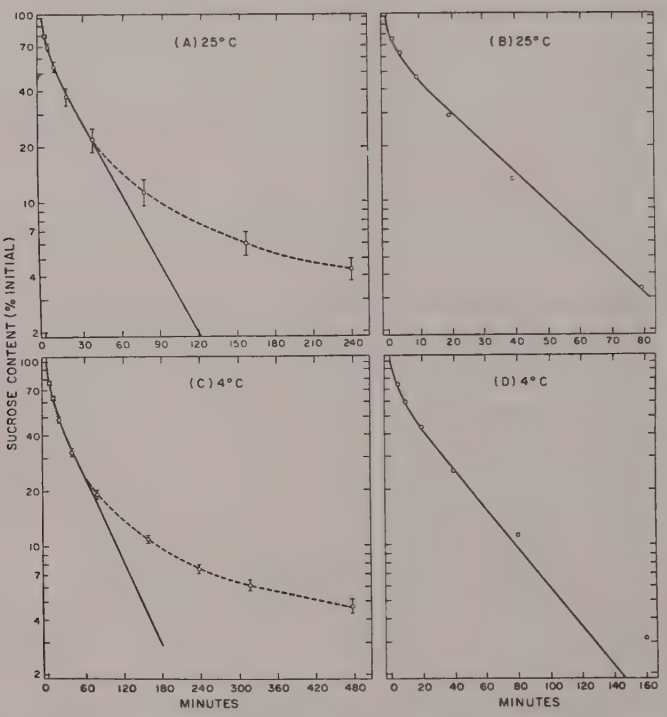

Fig. 5 (A, C) Decline of the sucrose content of desheathed sciatics by diffusion into Ringer's at 25° and 4°C. compared with the theoretical curve (solid line) for a homogeneous cylinder derived for k values estimated from the initial part of the curve (table 2). Each experimental point is the average of data from twelve nerves. (B, D) Corresponding corrected curves obtained by deducting a slow component estimated from the slope given by the last two points in A and C.

[4] The importance of antibiotics to prevent sucrose breakdown was shown by the apparent high temperature coefficient which was obtained with desheathed toad nerve before Penicillin and Streptomycin were employed. The earlier finding of a high temperature coefficient for sucrose diffusion in intact bullfrog nerve (Shanes and Berman, '53) is now believed to be an experimental artifact resulting from sucrose splitting.

seems justified that the "extracellular" space is not homogeneous, as reflected by the variation of the effective diffusion coefficient, although a large part of it may be roughly approximated by a single coefficient.

The temperature coefficients of sodium and chloride self-diffusion were pointed out to be somewhat greater than for self-diffusion. In the case of sucrose, the temperature coefficient is approximately the same as for free solution. Another important difference from the monovalent ions is in the relative magnitude of the effective diffusion coefficient for sucrose. As in the case of sulfate, it is smaller by a factor of two than would be anticipated from the relative mobilities and diffusibility in free solution. Hence, the rates of movement of the sugar and the divalent ion, relative to each other, are about that in aqueous solution. A common element therefore is present which reduces the diffusibility of these experimental agents but is lacking or less apparent for sodium and chloride.

The smaller sucrose space is suggestive of the absence of the molecule from the axon, consequently an extrapolation of the limiting slope, seen at late times in figure 5 A, C, to zero time presumably does not signify an axonal component. However, it may reflect the sugar proposed in the immediately preceding communication to be contained in the myelin sheath. The effect of such extrapolation therefore has been examined.

The corrected "extracellular" component, obtained as before by deducting the extrapolated slow stage, can be seen in figure 5 B, D to conform closely to homogeneous cylinder theory. The value of k obtained after such correction (table 2), is increased by only 30%, so that the diffusibility of sucrose in the nerve, relative to that of sodium and chloride, is little changed.

The extrapolation procedure for the disaccharide does not seem to have the same significance as for the monovalent ions, for the temperature coefficient of its slope, S (table 2), is substantially smaller than for S_{Cl} and S_{Na}. In the case of

the ions it is over three for a change of 21°C. and averages 3.4, while for the former it is 2.5, or about the value for the fast component of sodium and chloride. This may reflect the difference between sucrose diffusion from the myelin sheath and ionic transfer through nodes and internodes (see below).

In the absence of an analysis of the kinetics to be expected for molecules diffusing from structures like the myelin sheaths into the interfibrillar spaces, the significance of F for sucrose must be regarded as uncertain. With the interpretation applied to the monovalent ions, the disaccharide appears to come from a phase 10% of the total extra-axonal volume. Other considerations, however, suggest that the myelin water is closer to 40% (see table 5 in Shanes and Berman, '55).

THEORETICAL RESULTS

Sodium and chloride

An important problem in connection with the desaturation curves for Na^{22} and Cl^{36} is the extent to which, because of the delay in escape of radioactivity from the interstitial spaces, the limiting slopes and the zero time intercepts deviate as correct indices of average fiber penetrability and of the initial, relative axon content of radioactivity; conversely, an approximate indication is desirable of the error in assessing the value of k, the diffusion (or self-diffusion) coefficient of the extra-axonal spaces, when the extrapolated values of the slow component are simply subtracted from the total. These would be provided by a quantitative description of events.

Approximate derivation of desaturation curves. In the absence of a satisfactory explicit solution applicable to the conditions of our experiments, an approximate solution has been derived. For simplicity, the derivation for a homogeneous cylinder will be considered to describe the characteristics of the "extracellular" spaces; on the basis of the curves obtained after subtracting the extrapolated slow component for sodium and chloride, and from the complete curve for sucrose, we have seen that this may be true for at least 75%

of the extra-axonal space. The remainder, which may be derived from the myelin sheath, is neglected. A further simplification will be made in recognition of the initial rapid decline followed by the practically exponential fall of the theoretical desaturation curve for a homogeneous cylinder which is apparent from the figures. Such a curve would be given by a two phase system, say two coaxial cylinders, suddenly exposed to a non-radioactive solution; the outer cylinder, containing 30% of the radioactivity, loses its activity immediately, while the inner one behaves as though the contents remain well mixed and a hypothetical boundary governs the rate of loss, so that the decline in radioactivity is exponential. Thus, the desaturation curve for the extra-axonal region would be described by the ratio of the activity, Y_s, at time t to that, Y'_s, initially present:

$$Y_s/Y'_s = 0.3b + 0.7e^{-t/\tau}, \qquad (1)$$

in which $b = 1$ when $t = 0$, and $b = 0$ at $t > 0$. The time constant, τ, can be shown to be related to the mean diffusion coefficient, k, by

$$\tau = 0.173r^2/k, \qquad (2)$$

where r is the nerve radius. Schmidt ('53) has pointed out that the purely steric hindrance imposed by impervious fibers, uniformly distributed in the nerve and of the same diameters, may be expressed by the relation

$$k = Dgl. \qquad (3)$$

According to this, the effective diffusion coefficient, k, for the interstitial spaces is proportional to the free diffusion coefficient, D, but reduced by two factors: l, the ratio of the radius of the nerve to the actually longer effective radial path, and g, the ratio of the smaller effective area to the geometric area normal to the diffusion path. If the viscosity of the fluid in the interstitial spaces differs from an aqueous solution, still another correction factor would be necessary.

From this simplified approach, we may regard 30% of the fibers as subjected immediately to the applied external me-

dium, while the change to the external medium around the remaining 70% of the fibers tends to develop as an exponential function of time governed by a hypothetical boundary defined by equations 2 and 3.

Let us now replace the impervious fibers by slightly penetrable ones which do not alter g and l significantly. If the radioactivity remains uniformly distributed within each axon, and the axon boundary limits the rate of exchange, then upon immersion of the nerve in non-radioactive solution the activity of 30% of the fibers (i.e., those hypothetically exposed immediately to inactive solution) will decline exponentially. The remaining 70% of the system will behave effectively like two coaxial, membrane limited cylinders, the inner with a surface area, volume, and initial radioactivity equal to that of 70% of the fibers and the outer with properties described by equations 2 and 3 for the interstitial space.

The activity of the fibers undergoing a simple exponential decline can be described by

$$Y_1/Y'_1 = e^{-t/\tau_1} = e^{-P_1 t} \qquad (4)$$

where τ_1 is the time constant of decline, defined by the time in which $Y_1/Y'_1 = 1/e$, and P_1, the inverse of the time constant, is the "penetrability." P_1 is proportional to the "apparent permeability," but depends also on the ratio of the area to volume of the fibers (Harris and Burn, '49; Keynes and Lewis, '51; Shanes, '51, '52). The phrase "apparent permeability" is preferable to "permeability" since an electric field across the fiber boundary may contribute to the transfer of ions, accelerating the outflux of anions and decelerating that of cations (e.g., Goldman, '43), or the fibers may actively transport some of the experimental agents (Ussing, '49); hence, proper evaluation of permeability must correct for the existence of such potential differences or for the work done by the cell. This will be discussed later. Here it must be emphasized that in studies with radioisotopes such factors require specification of the direction of ion flux for a given "apparent permeability."

For the time being we shall use the phrase "apparent permeability" interchangeably with "permeability." It is most conveniently defined as the outflux per unit concentration of the phase losing radioactivity. Since the flux is expressed in mols/cm^2 min, the units are cm/min, which is consistent with the conventional definition of this cellular parameter. We may therefore write

$$P_i = m_{is} A_i / C_i V_i = h_{is} A_i / V_i \qquad (5)$$

We will now proceed to describe the remaining 70% of the system. The significance and dimensional units of the notation in equation (5) and in the succeeding equations are given first:

C = absolute concentration, assumed equal to thermodynamic activity (mols/cm^3)
h = apparent permeability (cm/min)
a = specific activity (counts/min mol)
V = aqueous volume (cm^3)
Y = aVC = absolute radioactivity (counts/min)
m = unidirectional flux; the sequence of subscripts gives the direction of flux (mols/min cm^2)
A = area (cm^2)

The subscript i is used for intracellular or cellular terms, the subscript s for interstitial characteristics, the subscript o for the medium, and primed symbols for values at zero time (i.e., at equilibrium or in the steady state). From this we have

$$b = V_i / V_s$$

P = "penetrability" of the fibers in an inward direction (min^{-1}) $= m_{si} A_i / C_s V_i$
P_2 = "penetrability" of the hypothetical extracellular boundary $= m_{so} A_s / C_s V_s$

$$Y'_i / Y'_s = V_i C'_i / V_s C'_s = b C'_i / C'_s = b P / P_i \qquad (6)$$

$$dY_i/dt = m_{si} a_s A_i - m_{is} a_i A_i \qquad (7)$$

$$= (m_{si}/C_s)(A_i/V_s) Y_s - (m_{is}/C_i)(A_i/V_i) Y_i \qquad (8)$$

$$= b P Y_s - P_i Y_i \qquad (9)$$

$$dY_s/dt = -dY_i/dt - m_{so} a_s A_s \qquad (10)$$

$$= -dY_i/dt - (m_{so}/C_s)(A_s/V_s) Y_s \qquad (11)$$

$$= -dY_i/dt - P_2 Y_s \qquad (12)$$

Equations 9 and 12 can be solved simultaneously with differential operators. This gives the desaturation curves for 0.7 of the intracellular and of the extracellular radioactivities:

$$Y_i/Y'_i = 0.5\ (1 + \alpha/\beta)e^{-(\alpha-\beta)t} + 0.5\ (1 - \alpha/\beta)e^{-(\alpha+\beta)t} \quad (13)$$

$$Y_s/Y'_s = 0.5(1 + \alpha/\beta - P_2/\beta)e^{-(\alpha-\beta)t} + 0.5(1 - \alpha/\beta + P_2/\beta)e^{-(\alpha+\beta)t} \quad (14)$$

$$\alpha = 0.5\ P_2(1 + P_1/P_2 + bP/P_2) \quad (15)$$

$$\beta = 0.5P_2\sqrt{(1 + P_1/P_2 + b\ P/P_2)^2 - 4\ P_1/P_2} \quad (16)$$

The sum of equations 13 and 14, after multiplication by factors giving relative values of Y'_i and Y'_s, will now describe the emergence of 0.7 of the total radioactivity. The two terms with the $\alpha - \beta$ exponent describe the slow component, those with the $\alpha + \beta$ exponent the fast component. The relation of these exponents to P_1 and P_2, which would have been the exponents in the absence of extracellular barriers or of permeable fibers, is not apparent by inspection. However, substitution of appropriate values for the constants demonstrates the order of errors involved. This will now be shown to be small.

The smaller the penetrability of the fibers relative to that of the extracellular spaces, the less will be the interaction of phases during the emergence of radioactivity. In the limit, when P_2 is infinitely large, P_1 alone governs the loss of radioisotopes. The worst situation, from the standpoint of such interaction, is therefore that in which P_1/P_2 is largest. Considering S/k, from table 2, to be proportional to P_1/P_2, we find the poorest of the sodium and chloride curves to be those describing the emergence of Na^{22} at room temperature from Ringer's or NaCl equilibrated nerves. These two instances will therefore be considered.

We may approach the problem by assuming that our estimates of the penetrabilities P_1 and P_2 from the experimental curves are roughly correct and then seeing how closely the curves calculated from these values conform to the actual ones. Therefore

$$P_1 = S$$

and

$$P_2 = 1/\tau = 5.8\ k/r^2,$$

which may be solved with the data from table 2. Since $P = P_1 C'_i / C'_s$, an estimate of C'_i, the intracellular concentration, is needed. In the case of NaCl treated nerves, the equality of the Na^{22} and water spaces (Shanes and Berman, '55) signifies $C'_i = C'_s$, hence $P = P_1$. For Ringer's soaked nerves, we take the aqueous volume of the axon as equal to 0.3 ml/gm (Shanes and Berman, '55); accepting $F = 16\%$ as a roughly correct estimate of the fiber content of sodium, we calculate from the average total sodium of 73 μM/gm (Shanes and Berman, '55) $C'_i = 0.4\ C'_s$, or $P = 0.4\ P_1$. $V_i/V_s = b$ will be taken as 0.6, since with $V_i = 0.3$ ml, $V_s = 0.5$ ml.

These relations therefore permit the tabulation of the parameters for equations 13–16 which are given in table 3. The final equations are also given. Comparing P_1 with $\alpha - \beta$, and P_2 with $\alpha + \beta$, we see that they differ by less than 4%; hence, if our derivation is a reasonable approximation of events, the slopes of the slow components for sodium and chloride are a close measure of fiber "permeability" and the k values in table 2 of the effective diffusion coefficients in the extra-axonal space.

Because P_1 and $\alpha - \beta$ are so similar, the term describing 30% of the fiber radioactivity which has P_1 in the exponent has been lumped with the derived term for the remaining 70%, giving the first term in equations (4b) and (8b) in table 3. The factors in these first terms give the theoretical zero time intercepts, which are to be compared with F in table 2. Thus, for Ringer's soaked nerves the intercept is 17.6% compared to F of 16%, considered to be the correct initial percentage of radioactivity in the fibers. And for NaCl soaked preparations the calculated value is 32.9% compared to 30%. Percentagewise the error in F is the same — about 10% — for both cases.

On the basis of our derivation, therefore, little error is involved in accepting the slope of the slow components of the experimental curves as a measure of apparent fiber permeability, and the zero time intercept as the fraction of

total activity initially contained in the fibers. The negligible interaction of the two phases has also been demonstrated independent of the above assumptions by the agreement of the change in F with that to be expected from the uptake of sodium in NaCl treated nerves.[5]

TABLE 3

Parameters and equations describing the emergence of Na^{22} from Ringer's equilibrated and NaCl equilibrated nerves

PARAMETER	UNITS	RINGER'S NERVE	NaCl NERVE
P_1	min^{-1}	9.7×10^{-3}	7.7×10^{-3}
P_2	min^{-1}	0.181	0.138
P	min^{-1}	3.88×10^{-3}	7.7×10^{-3}
b		0.6	0.6
α	min^{-1}	9.66×10^{-3}	7.52×10^{-3}
β	min^{-1}	8.71×10^{-3}	6.78×10^{-3}
$\alpha - \beta$	min^{-1}	9.5×10^{-3}	7.4×10^{-3}
$\alpha + \beta$	min^{-1}	0.184	0.143
$0.5(1 + \alpha/\beta)$	%	105.5	105.5
$0.5(1 - \alpha/\beta)$	%	— 5.5	— 5.5
$0.5(1 + \alpha/\beta - P_2/\beta)$	%	1.5	3.5
$0.5(1 - \alpha/\beta + P_2/\beta)$	%	98.5	96.5

Ringer's:
$$Y_1/Y'_1 = 105.5e^{-0.0095t} - 5.5e^{-0.184t} \quad (1a)$$
$$Y_s/Y'_s = 1.5e^{-0.0095t} + 98.5e^{-0.184t} \quad (2a)$$
$$Y_T/Y'_T = 0.16\, Y_1/Y'_1 + 0.84\, Y_s/Y'_s \quad (3a)$$
$$= 18.2e^{-0.0095t} + 81.8e^{-0.184t} \quad (3b)$$
$$(Y/Y')_{t>0} = 0.7\, Y_T/Y'_T + 0.3(16)e^{-P_1 t} \quad (4a)$$
$$\cong 17.6e^{-0.0095t} + 57.2e^{-0.184t} \quad (4b)$$

NaCl:
$$Y_1/Y'_1 = 105.5e^{-0.0074t} - 5.5e^{-0.143t} \quad (5a)$$
$$Y_s/Y'_s = 3.5e^{-0.0074t} + 96.5e^{-0.143t} \quad (6a)$$
$$Y_T/Y'_T = 0.3\, Y_1/Y'_1 + 0.7\, Y_s/Y'_s \quad (7a)$$
$$= 34.1e^{-0.0074t} + 65.9e^{-0.143t} \quad (7b)$$
$$(Y/Y')_{t>0} = 0.7\, Y_T/Y'_T + 0.3(30)e^{-P_1 t} \quad (8a)$$
$$\cong 32.9e^{-0.0074t} + 46.1e^{-0.143t} \quad (8b)$$

From these considerations, therefore, the coefficients given in table 2 are regarded as approximately correct measures of the averaged properties of the axons, their membranes, and the interstitial spaces.

Effects of fiber size. The above derivation, as in previous analyses (e.g., Schmidt, '53), requires that the fibers all be

[5] See footnote 1, page 207.

of uniform geometry or that an "average fiber" describe the properties of the whole population of fibers. In view of the wide range of fiber diameters characteristic of nerve (e.g., Gasser and Erlanger, '27; Sanders, '48), including those from the toad (Longley, '53), an examination of the possible role of this parameter, particularly in relation to the structural elements of the fibers, appears desirable. This must include a consideration (a) of the myelin sheath as a phase containing electrolyte, (b) of the relative importance of the nodes and internodes in controlling ionic exchange, and (c) of the sizes of interstitial compartments enclosed by fibers of different sizes.

Taking the last item first, we may note that even if the distance between adjacent fibers, measured from their peripheries, were constant, the surface available for escape (i.e., the distance between fibers), relative to the interstitial volume enclosed by a small group of fibers, would vary with the dimensions of the surrounding fibers. The time constant of emergence of ions or molecules from such fiber-bound compartments would necessarily depend inversely on the area to volume ratio, which may account in part for the lack of uniformity of the diffusion or self-diffusion coefficients seen with sucrose and the fast components of the ions. The temperature coefficient in this case would be the same as for free diffusion, which is not quite the situation for the slow component of sucrose escape.

Similarly, the variation of the surface to volume ratio of the fibers with size would be expected to prevent the slow component of the whole nerve from being a simple exponential (see equation 5). The strict exponential decline actually obtained demonstrates the presence of compensatory elements. Whether these are inherent in the peculiarities of fiber structure or in the nature of the distribution of fiber sizes will now be examined.

First, the relative importance of the nodes and internodes merits consideration. Measurements on single fibers have shown that while the resistivity (i.e., the ohms for a square

centimeter of surface) is much greater for the internode, its surface area is correspondingly greater, so that its net resistance in a large fiber is only about two and a half times as great as for a node, viz., 200 as compared to 80 megohms (Tasaki, '53; Stämpfli, '54). If the inverse of resistance — the conductance — is proportional to ion transferability (Hodgkin, '51), the internode may carry almost as many ions as the node, but of course at a substantially lower density.[6] The demonstration of the dependence of internode conductance on Ringer's salinity (Tasaki, '53) adds to the likelihood that ion exchange can proceed through the myelin.

From this standpoint, then, a large "hot" myelinated fiber subjected to a radioisotope-free medium loses activity through the nodes and internodes. For both types of loss it can be shown that the fiber dimensions are sufficiently small so that the axon activity would remain essentially uniform.

Now let us examine the dependence of node and internode penetrability, P, on fiber size. As described previously,

$$P = hA/V, \tag{17}$$

but for a given fiber h and A are the apparent permeability and area of the structure involved and V is the volume of the axon. All are referred to a unit length of fiber.

In the case of the internode, the mean surface area will be defined by

$$A_I = 0.5\pi(W + w), \tag{18}$$

where W is the total diameter and w is the diameter of the axon.

The permeability of the sheath is probably a function of thickness. We therefore write

$$h_I = k_M/0.5(W - w), \tag{19}$$

[6] This is consistent with the ineffectiveness of experimental agents applied to the internode (Tasaki, '53) since the low permeability there and the high diffusibility in the axon would combine to prevent appreciable accumulation at the axon-myelin interface, where a functional membrane might be present.

in which k_M is the diffusion coefficient of myelin. Also

$$V = 0.25\pi w^2, \tag{20}$$

which is the same for internode or node. Over a wide range of sizes of fresh amphibian fibers, the data of Schmidt and Bear ('37) indicate

$$W = 1.43\ w. \tag{21}$$

Substituting in equation *17*, we obtain the relation

$$P_I = 23\ k_M/w^2. \tag{22}$$

Thus, if the diffusion coefficient of myelin is the same in different fibers, P_I is inversely related to the square of axon diameter, which makes it strongly dependent on fiber size.

The penetrability of the nodes is also very sensitive to axon diameter. Thus, Rushton's analysis ('51) leads to the conclusion that the area of a node is governed by

$$A_N = Cw^2/x, \tag{23}$$

where x is the distance between nodes and C is a proportionality constant. Data obtained by Tasaki et al. ('44) for living bullfrog fibers indicate that

$$x = 150\ W \cong 210\ w. \tag{24}$$

The relative dimensions of the nodes of large fibers (de Renyi, '29; Stämpfli, '54) permit an evaluation of C, which leads to

$$A_N = 10^{-4}\ w \tag{25}$$

in cm^2 when w is in cm. Since all parameters are expressed per unit length of fiber, A_N must be multiplied by the number of nodes per centimeter, which is 1/x. V is the axon volume, hence

$$P_N = h_N A_N/Vx \cong 0.6 \times 10^{-6}\ h_N/w^2. \tag{26}$$

These formulations of the penetrability of the nodes and internodes in a centimeter of nerve tell us that if these structures solely determine the emergence of radioactivity and are governed by the same size relations as in the frog, then unless the fibers are very numerous in a limited range of axon diameters — which is unlikely if the toad fibers resemble

those in the frog (Gasser and Erlanger, '27) — the slow component is not likely to be a simple exponential. Since this is contrary to the actual experimental situation, factors may not have been considered in the above approach which compensate for the effect of diameter. What these may be will now be discussed.

Structural elements of the fibers. Up to this point the axoplasm has been considered as the major site of origin of the slowly emerging fraction of the radioisotopes, and the nodes and internodes as the chief barriers to diffusion from the axon. The kinetics of the slow and fast components may also be related (1) to an exchangeable fraction of radioisotope bound to protein or lipid components of the trunk (Manery et al., '38; Manery and Haege, '41; Christensen and Hastings, '40), (2) to the myelin sheaths as a discrete phase, and (3) to other sheathing elements, such as Schwann's sheath and the connective tissue sheath of Henle (de Renyi, '29).

The observations by de Renyi ('29) demonstrate that the last mentioned sheaths are not uniform, the connective tissue sheath being denser at nodes than at internodes, and Schwann's sheath more firmly adherent at the nodes. The significance of these histological factors for resistance and flux measurements remains to be determined. They may therefore contribute to reducing the sensitivity of penetrability to diameter. Neither can be regarded as a major barrier, since this would be associated with the exclusion of sucrose from contained structures, including the myelin, which available evidence indicates is accessible to the disaccharide (Shanes and Berman, '55).

In view of the likelihood that the aqueous phase of the myelin sheaths is in equilibrium with the medium, it becomes pertinent to inquire regarding the extent to which the slow component may be attributable to ions coming from the myelin sheaths.

As a first approximation, ions derived solely from the sheaths would follow the time course to be expected for the

loss from a plane sheet (Hill, '28). In this case, except for an initial more rapid release involving about 15% of the total activity, most of the exchange would follow an exponential time course with a time constant (1/S) inversely related to the square of the sheath thickness. Since myelin thickness is directly related to fiber diameter, we emerge with a situation which again would not conform with the finding that in preparations containing fibers of a wide range in diameter the slow component follows an exponential time course. However, in this case, too, unrecognized compensatory factors might reduce the dependence of penetrability on diameter.

A more serious objection to the view that the slow component of the desaturation curves is derived from the myelin sheaths becomes apparent when the effect of NaCl treatment on Na^{22}-Na^{23} exchange is examined. Thus, if in Ringer's soaked nerves Na^{22} emerges only from the myelin, plane sheet theory can be shown to give a final slope with the time constant

$$\tau_P = 0.39B^2/k_M, \qquad (27)$$

where B is the sheath thickness and k_M is the diffusion coefficient of the myelin. If, as a result of NaCl treatment, the axons have gained sodium, Na^{22} will now emerge first from the myelin followed by that from the axon. The final slope of the outflux will be governed by the same approximate equation as used for epineurium controlled diffusion (Shanes, '54b), viz.,

$$\tau_A = 0.25wB/k_M. \qquad (28)$$

The two time constants are more easily compared by expressing B in terms of w, given by

$$B = 0.5(W - w) \cong 0.22w. \qquad (29)$$

Hence,

$$\tau_P = .019\ w^2/k_P \qquad (30)$$

$$\tau_A = 0.05w^2/k_P \qquad (31)$$

Consequently the emergence of ions from the axons will be slower by a factor of 2.5 than from the sheath, hence the

slope for sodium emergence from Ringer's treated nerves should have been 2.5 times greater if the major site of origin were different in this way from that in NaCl treated preparations. In the absence of such a substantial alteration, it appears unlikely that the sheath was the source of the slow sodium component in the Ringer's preparations. If there is a sheath component, it is to be sought in the fast rather than in the slow component; the deviation we have noted at later times from homogeneous cylinder theory (figs. 2 B, D and 3) has already been pointed out as a possible consequence of such a myelin component.

With regard to binding as a cause of the slow emergence, this probably can be dismissed for the extracellular ions. Thus, the exchange of Cl^{36} in tendon, where chloride binding by the connective tissue has been demonstrated (Manery et al., '38), is diffusion limited under *in vitro* conditions (Cotlove, '54). In the case of sodium, the equality of the slopes of the slow components of Ringer's and NaCl treated preparations again appears to rule out the possibility that in Ringer's the slow component is derived from "bound" extracellular sodium unless, of course, the rate constants are fortuitously identical.

Ion distribution in desheathed toad nerve. Our interpretation of the significance of the sucrose space and F leads to the electrolyte balance sheet shown in table 4. This is considered more exact than the tentative one presented on the basis of space data (Shanes and Berman, '55). The chief difference is in the substantially smaller axonal contents predicted from the values of F; our analysis has shown that even this may be 10% too high (see table 3).

Taking the values calculated directly from F, we obtain axon concentrations of both sodium and chloride which are relatively high — comparable to those reported for the squid giant fiber (Hodgkin, '51). If the 71 millivolt resting potential, estimated indirectly for frog fibers (Huxley and Stämpfli, '51), is correct and applicable to the toad, the potassium and chloride concentrations are substantially greater

than are to be anticipated for passive distribution according to electrochemical gradients.

Our approach leads to a small quantity of sodium and chloride which is unassigned. The small amount involved may be within the uncertainties of measurement or of our interpretations. The possibility of "binding" cannot be overlooked, however. Our preceding communication has discussed in detail how interfibrillar connective tissue might contribute

TABLE 4

Estimated ion distribution in 1 gm wet weight of Ringer's equilibrated desheathed toad nerve

REGION	ION CONTENT			WATER CONTENT	ION CONCENTRATION		
	K	Na	Cl		K	Na	Cl
	μM	*μM*	*μM*	*ml*	*μM/ml*	*μM/ml*	*μM/ml*
Total [1]	38.6	73.1	68.7	.795	..	..	..
Axons	37.7	11.7 [2]	7.6 [3]	.28 [2]	135	42	27
Myelin	0.32	20.5	20.9	.19 [4]	1.7	108	111
Interfibrillar	0.55	34.6	35.2	.32 [4]	1.7	108	111
R [5]	..	6.3	5.0	..	..	...	...

[1] Shanes and Berman ('55).

[2] Difference obtained by subtracting from the total water content the average sucrose space (table 1).

[3] F × total.

[4] For derivation see Shanes and Berman ('55); this aqueous phase is assumed to be Ringer's.

[5] Remainder.

to half of the excess chloride and the cephalin to considerably more than the excess sodium.

Table 4 describes the situation for desheathed nerves at least 16 hours after removal from the animal. The remarkable stability of these preparations with respect to weight, potassium content, and spikes during this period (Shanes, '54a) and for 24 hours or more longer (Shanes and Berman, '55), makes it the probable one for fresh preparations. Final proof of this is contingent on *in vivo* experiments with plasma concentrations carefully controlled for sufficient periods of time.

Ion permeability in desheathed toad nerve. The estimates of ion permeability are contingent on the model ascribed to the nerve fibers. Although the substantial dependence of the penetrability of internode and node on fiber diameter to be expected theoretically appears to be out of keeping with the configuration of the desaturation curves, in the absence of a more acceptable basis of calculation, the relations established for them will be considered to give the orders of magnitude of the permeability coefficients. The mean fiber diameter (W) will be taken as 10 μ, the axon diameter (w) 7 μ. Substituting these values and the data for $S(=P_I, P_N)$ in equations 22 and 26, we obtain the "apparent" permeability coefficients when either the internodes or nodes are assumed to govern ionic exchange. These are given in table 5. As pointed out previously, "apparent" is used since correction may still be necessary for the contribution of an electric field and active transport.

The constant field theory (Goldman, '43; Hodgkin and Katz, '49) provides relations which correct "apparent" permeability for membrane potential. Designating the "true" permeability by *j*, we have, for monovalent anions,

$$j_A = (RT/EF)(1 - e^{-EF/RT})\, h_A,$$

and for monovalent cations,

$$j_C = (RT/EF)(e^{EF/RT} - 1)\, h_C.$$

E is the potential difference across the membrane and R, T, and F are the usual thermodynamic terms. If the 71 millivolt membrane potential estimated for frog fibers (Huxley and Stämpfli, '51) is employed, the correction factor for chloride is found to be 0.34 and that for sodium 5.2. Thus, the true permeability for chloride is about 1/3 that obtained by neglecting the membrane potential, which accelerates anion outflux; conversely, for sodium it is five-fold greater. Hence sodium and chloride permeabilities may differ by a factor of 15 more than indicated by the apparent permeabilities.

Across the myelin sheath, however, no potential difference may exist. Thus, Tasaki ('52), with microelectrodes, observed

no potential difference between the myelin and the external solution until the sheath was completely punctured. This may indicate the restriction of the potential difference to an inner layer. Similarly, Schwann's and Henle's sheath at the node may modify the relationships there. Hence, the correction factors based on membrane potential cannot be applied with certainty until the site representing the major barrier to diffusion or exchange can be specified.

The contribution of active sodium transport to the apparent permeability should be demonstrable by a decrease in outward

TABLE 5

Estimates of the apparent permeability coefficients, for an average fiber 10^{-3} cm in diameter, when permeation is governed by the internodes (equation 22) and the nodes (equation 26)

ION	TEMPERATURE	INTERNODE [1]		NODE [1]	
		A	B	A	B
	°C.	$10^7 \times$ cm/min		$10^3 \times$ cm/min	
Sodium [2]	25	12	3.4	7.1	5.1
	4	3.3	0.94	3.0	1.4
Chloride	25	7.2	2.1	4.3	3.1
	4	2.2	0.63	1.3	0.93
Sulfate	25	1.0	0.28	0.6	0.42

[1] B is obtained from A on the assumption that node and internode contribute to ion interchange inversely as their estimated resistances (Stämpfli, '54).

[2] Mean values of P_{Na} for Ringer's and NaCl soaked nerves employed.

flux in the absence of metabolism. Data now at hand (unpublished) show that a combination of inhibitory conditions (anoxia, iodoacetate, dinitrophenol and eserine) do not alter sodium outflux. That metabolism has been modified is demonstrated by the marked reduction in potassium influx (to about one-third) by anoxia with iodoacetate alone; potassium outflux also is increased somewhat. These findings support the earlier conclusion (Shanes, '51, '52) that potassium rather than sodium is more directly linked to active transport processes in amphibian sciatic nerve.

Ionic fluxes. The evaluation of the absolute outflux, given by the product of h and concentration, does not require in-

formation on the membrane potential and the transfer mechanism, but still depends on a knowledge of the intracellular thermodynamic activity of the ion in question. For example, if some binding has occurred, the thermodynamic activity involved would be smaller than indicated from the total analytical concentration.

If the concentrations and h values as given in tables 4 and 5 are employed, sodium and chloride outfluxes at 25° are between 6 and 50 $\mu\mu M/cm^2$ min at the internodes and between 0.08 and 0.4 $\mu M/cm^2$ min at the nodes. The resistance estimated for the internode is 0.16×10^6 ohm cm^2 and for the node (on the basis of an area of 10 μ^2) about 8 ohm cm^2 (Stämpfli, '54).

In frog sartorius muscle the outflux of sodium and chloride is about 600 $\mu\mu M/cm^2$ min and the membrane resistance 5000 ohm cm^2 (Hodgkin, '51). Thus, the estimated flux through muscle fibers is 10 to 100 times greater than that for the internode, which corresponds well with the 30-fold greater internode resistance. Similarly, the estimated outflux across the nodes is between 100 and 600 times greater than across the muscle membrane, which is in keeping with the 600-fold greater resistance of the muscle membrane. The relative outfluxes therefore correspond inversely with the resistances, as would follow from the dependence of the electrical parameter on flux in the steady state or at equilibrium (Hodgkin, '51).

Sucrose and sulfate

The k values of these radioisotopes compared with their free diffusion coefficients were half as great as the corresponding figures of sodium and chloride. The possible origin of this difference will now be considered.

It will be recalled that the effective diffusion coefficient, k, depends on the relative decrease in effective area normal to radial diffusion, and the longer mean path introduced by the presence of the fibers in the nerve trunk (equation 3). Obviously, then, the values of k will correspond to those in free solution only as long as the permeability or impermeability

of the fibers makes them a barrier to the same degree for different substances.

Since the axons appear to be impermeable to sucrose, and have the lowest permeability to sulfate, they may indeed be expected to be a greater barrier (i.e., Schmidt's gl smaller) to those experimental agents than to the monovalent ions. The relative permeabilities in myelin would likewise play a part. If these views are correct, lower temperatures should lead to better agreement of the effective diffusion coefficients by rendering the fibers less permeable to the monovalent ions.

TABLE 6

The effect of temperature on the product of the relative diffusion (or self-diffusion) coefficients of the "extracellular" spaces and the inverse ratio of corresponding free diffusion (or self-diffusion) coefficients

TEMPERATURE	IONS OR MOLECULES		k_xD_y/k_yD_x
°C.	x	y	
25	Cl	Na	1.05
4	Cl	Na	0.88
25	Cl	Sucrose	2.4
4	Cl	Sucrose	1.4
25	Cl	SO_4	2.3
25		SO_4	1.5
4	Cl		
25	Sucrose	SO_4	0.9

This is shown to be the case in table 6. If the ratio of the coefficient of a reference monovalent ion to that of another diffusible substance is multiplied by the inverse ratio of their free diffusion coefficients, the product will be one if the k's have the same relative magnitudes as the D's. In table 6 this can be seen for the monovalent ions, which have nearly identical permeabilities. For sulfate and sucrose the discrepancy relative to chloride at room temperature is about 2.5-fold, while at low temperatures the agreement is much better. Presumably, a still lower temperature would give a product close to one. The proximity of the sucrose-sulfate

product to one at room temperature may be taken to indicate that for permeabilities as low as for sulfate the nerve fibers are practically equivalent to impervious rods.

On qualitative grounds, the approach presented accounts for the relatively high temperature coefficient for the k values of the monovalent ions and the low one for sucrose. Thus, in the case of the disaccharide, which presumably is largely restricted to the extrafibrillar spaces, diffusion occurs chiefly through the water contained in this region and hence has the same temperature coefficient. In the case of the monovalent ions, however, two paths essentially in parallel are involved, one as for sucrose *between* the fibers, the other *through* the fibers. Since the resistance to exchange across fiber membranes has a temperature coefficient of over three (S values in table 2), the effect of temperature on k for the monovalent ions would lie intermediate to the two extremes.

A more precise analysis of this situation may be possible by setting up relationships on a basis similar to that derived by Cole (see Cole and Curtis, '50) for the conductance of parallel cylinders in a conducting medium.

DISCUSSION

The foregoing analysis makes evident the numerous factors which must be weighed in interpreting the kinetics of diffusion or exchange (self-diffusion) in a structure as complex as a desheathed vertebrate nerve trunk.[7] While from this standpoint other preparations are preferable, the toad sciatic is superior in the strictly steady state or equilibrium conditions that it provides.

The time course of decline of the nerve radioactivity closely resembles that which has been described in muscle and other biological systems, where an initial fast component has been

[7] No consideration has been given to the possibility that radioisotopes within the capillaries may also contribute to the desaturation curves. This is justified by the small volume these usually constitute; thus, in frozen dried sections of rat nerve, the cross-sectional areas of blood vessels represent between 0.9 — 1.8% of the total (Birren, personal communication).

attributed to the extracellular phase and a secondary slow component to the cells. However, the present study has shown that a molecule like sucrose, which by virtue of its restricted uptake appears to be localized in the "extracellular" spaces, nevertheless emerges in a non-exponential fashion which might likewise be attributed to at least two phases. While in nerve this may actually reflect two extra-axonal phases, controls of this type are required in other systems before current interpretations of desaturation curves can be regarded as valid.

The emergence of a major part, but not all, of each of the diffusible agents from the extracellular spaces has been shown to follow the theory for a homogeneous cylinder. This has provided average values of the diffusion or self-diffusion coefficients for sodium, chloride, sulfate and sucrose. The temperature coefficient for the last indicates diffusion chiefly in the aqueous phase of the "extracellular" spaces, while that for the first two indicates the involvement of a phase with a greater temperature sensitivity. This is shown to be consistent with the view that the permeability of the fibers to the monovalent ions provides an additional path for diffusion which is essentially unavailable to sucrose, namely, the fibers themselves. Further evidence for this is seen in the two-fold greater diffusion coefficients calculated for the monovalent ions over and above their diffusibilities in free solution relative to sucrose and sulfate and in their greater dependence on temperature.

The relation of the slow component of the desaturation curves to a specific structural component of the fibers is less certain. The simple exponential decline of activity at late times is not to be expected for a wide range of fiber sizes resembling that in frog nerve. Unfortunately, this and other histological data on toad fibers are unavailable. When relationships established for frog fibers are applied, they are found to lead to time constants of exchange or diffusion which vary as the square of the fiber diameters. Unidentified compensatory factors may be present which reduce the sensitivity

to fiber size, and thus may lead to the simple exponential decline seen in our multifibered preparations. Before these difficulties can be resolved, the validity of applying histological information from the frog to the toad remains to be examined. The important difference already noted in the epineuria and perineuria of these two species (Shanes, '53, '54a) demonstrates the need for caution in this regard.

In addition to the lack of histological data on the toad, procedures are not presently available to demonstrate in the case of the anions, as could be done for sodium, the similarity of the kinetics under conditions known to cause ion uptake. Thus, the interpretation of F_{Cl} and F_{SO_4}, and S_{Cl} and S_{SO_4} as measures of axonal content and fiber permeability to chloride and sulfate rest on an analogy with sodium. The possibility of other sites, for example the myelin itself, cannot be dismissed at present, although the assumption of chloride in the axoplasm leads to fluxes consistent with available resistance data.

Still another element of uncertainty hinges on the phase or phases characterized by the "sucrose space." Its smaller value than for chloride may indicate (a) "bound" chloride and/or (b) intracellular chloride. Both possibilities are suggested by F_{Cl} being smaller than can be accounted for by the difference in chloride and sucrose spaces. At present the most acceptable view is that the aqueous phase of myelin is chemically part of the "extracellular" space and is included in the sucrose space.

With current information, the following tentative description of desheathed toad nerve appears most satisfactory. The geometrical volumes of the axons, myelin sheaths and interstitial spaces are all about equal. The aqueous content of myelin is of the order of 60% of the myelin weight and part of the extracellular space. Sodium and chloride concentrations in the axon are appreciable — of the order of 40 and 30 μM/ml, respectively. Both node and internode probably are sites of entry and exit for these ions.

SUMMARY

The time course of emergence of a variety of radioisotopes, following a preliminary equilibration in "hot" solutions, has been examined in desheathed toad nerve.

The diffusion of C^{14} labelled sucrose conforms largely to that to be expected from a homogeneous cylinder, i.e., most of the decline can be described by a single effective diffusion coefficient which is about 1/30 of that for free aqueous diffusion. However, about 20% of the sucrose emerges still more slowly, demonstrating that homogeneous cylinder theory represents only an approximation of events in the "extracellular" spaces. The temperature coefficient of the major component of escape approximates that for free diffusion, while the smaller component has a somewhat larger coefficient.

The loss of Na^{22} and Cl^{36} by exchange with the nonradioactive isotopes (Na^{23} and Cl^{35}), and of $S^{35}O_4$ by diffusion, also occur in two distinct stages — an initial rapid one followed by a smaller slow one which is exponential. The temperature coefficient of the first component is closer to that of free diffusion — ca. 2.5 for a change of 21°C. — while that of the second is considerably greater — ca. 3.4 for the same temperature change.

The uptake of sodium by nerves equilibrated in NaCl is reflected by the larger amplitude of the exponential decline. The increase in amplitude is shown to be an exact measure of sodium uptake, from which it is concluded (a) that the intercept obtained by extrapolation of the slow component to zero time gives the fraction of intrafibrillar sodium and (b) that its slope is a measure of outwardly directed fiber penetrability. The slope of the slow decline on semi-log coordinates is unchanged by NaCl treatment, hence this penetrability is unaltered and the rate of emergence is proportional to the intracellular sodium concentration; the inward penetration of sodium is permanently increased, however.

Most of the rapid emergence, which is from the extra-axonal region, can be described by diffusion theory for a

homogeneous cylinder; divergence from this is ascribed (a) to the emergence of particles from the myelin sheaths, which are chemically part of the "extracellular" space, (b) to the numerous compartments of different surface to volume ratios into which the interstitial space is divided by the different sized fibers, and (c) to the different resistance offered by the fibers to diffusion and self-diffusion by virtue of their different permeabilities to the various experimental agents used.

An analysis of the kinetics of radioisotope exchange indicates that ionic transfer through the fiber membranes in desheathed toad nerve is little interfered with by the resistance to diffusion imposed by the extrafibrillar spaces. Both node and internode may contribute to exchange. The relation of fiber size to fiber structure, as found in the frog sciatic, suggests a greater dependence of penetrability on diameter than is evident in the desaturation curves. Unless the relationships of toad fibers are quite different from those in the frog, compensatory factors, such as might be introduced by peripheral fiber sheaths, may account for this apparent discrepancy.

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SUCCINO-CYTOCHROME C REDUCTASE ACTIVITY OF TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L)

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In the intermediate metabolism of insects, as in other animals, the succinoxidase system is of vital importance. Although this enzyme system has recently been studied in the muscle of the cockroach (Barron and Tahmisian, '48, and Harvey and Beck, '53) as well as of flies (Watanabe and Williams, '51, and Sacktor, '53), its distribution in the other tissues of an insect had not been determined. An approach to related problems in the comparative physiology of insect tissues was, however, reported previously. It was found by Sacktor and Bodenstein ('52) that different tissues of the American cockroach have different cytochrome *c* oxidase activities, and that these enzymatic activities correspond with the extent of tracheation of these tissues (Day, '51). Also, the tissues were distinct in their ability to dephosphorylate adenosine triphosphate (Sacktor et al., '53). It was discovered additionally that the enzymatic activity of several tissues was significantly correlated with the sex of the animal. These observations support a previous report by Barron and Tahmisian ('48) of enzymatic differences between male and female muscle homogenates. Since the succino-cytochrome *c* reductase system is also involved in the respiratory metabolism of the insect, it is of interest to examine its distribution with reference to the evidence already cited that each tissue has a specific metabolic potential. Accordingly, the present communication is concerned with a comparative study of the

capability of the various American cockroach tissues, male and female, to reduce cytochrome *c* in the presence of succinate.

EXPERIMENTAL PROCEDURES

Adult male and female roaches were dissected in 0.9% KCl and the desired tissues were removed as described previously (Sacktor and Bodenstein, '52). The tissues, immediately prior to enzymatic assay, were homogenized for 30 seconds in cold KCl solution with a Potter-Elvehjem homogenizer. The quantity of KCl used in homogenization was as follows: muscle, 2.0 ml; Malpighian tubes, nerve cord or brain, 0.5 ml; and for the remaining tissues, 1.0 ml. Separate determinations were made on the tissues from each of 10 roaches. Because of the small amount of tissue available from one roach, the separate tissues of two roaches were pooled for the experiments with brain and Malpighian tubes. Thus, 20 roaches were used for the 10 determinations of the enzymatic activity of these tissues.

Succino-cytochrome *c* reductase activity was measured essentially as reported earlier (Sacktor, '53). This method is based on the rate of reduction of cytochrome *c* under standard conditions in the presence of cyanide to inhibit cytochrome *c* oxidase. The reduced cytochrome *c* concentration was determined with a Beckman model DU spectrophotometer and calculated according to Cooperstein et al., ('50). The reaction mixtures, per milliliter, had: 0.02 μM cytochrome *c;* 1 μM KCN; 15 μM succinate; 25 μM tris (hydroxymethyl) aminomethane buffer, pH 7.4; 0.2 ml of tissue homogenate (except muscle, where but 0.1 ml. was used); and 0.9% KCl to a final volume of 3.0 ml. With this procedure for enzymatic assay, the rate of reduction of cytochrome *c* is directly proportional to the amount of enzyme present. Furthermore, the reduction of cytochrome *c* is insignificant in the absence of succinate or homogenate.

Protein was determined by an adaptation (Sacktor et al., '53) of the method of Lowry et al. ('51).

RESULTS

The relative capabilities of various cockroach tissues, male and female, in reducing cytochrome *c* in the presence of succinate are shown in table 1. In general, based on their activity, they can be grouped into three categories. These are (1) those with greatest activity: muscle and hindgut; (2) those with moderate activity: fat body, midgut, foregut and Malpighian tubes; (3) those of low activity; brain and nerve cord. Since the hindgut is concerned with the resorption of

TABLE 1

Succino-cytochrome c reductase activity of American cockroach tissue

TISSUE	ACTIVITY [1]/MG PROTEIN				OXIDASE: REDUCTASE RATIO
	Male		Female		
	Mean ± S.E.		Mean ± S.E.		
Muscle	.177	.020	.045	.005	6:1
Foregut	.034	.003	.054	.013	9:1
Midgut	.045	.007	.038	.005	8:1
Hindgut	.125	.018	.066	.007	5:1
Malpighian tubes	.054	.010	.029	.005	4:1
Fat body	.063	.007	.049	.010	...
Brain	.033	.006	.026	.008	16:1
Nerve cord	.033	.005	.019	.002	10:1

[1] $\frac{\Delta \log [\text{cytochrome } c]}{\Delta t \text{ (min.)}}$. Each datum is the average of 10 determinations. The values used for cytochrome *c* oxidase activity were from data reported by Sacktor and Bodenstein ('52).

materials from the alimentary canal, the high rate of reductase activity found in this tissue in conjunction with its large metabolic potential, as indicated by its tracheation and cytochrome *c* oxidase activity, further supports the hypothesis that the resorption mechanism requires a large expenditure of energy (Sacktor and Bodenstein, '52). This energy can be obtained from oxidative processes. It had been suggested previously that the fat body also may be the site of extensive cellular metabolism (Bodenstein, '53, and Sacktor et al., '53). The present data are in furtherance of this view.

Table 1 also indicates that the succino-cytochrome *c* reductase activity of a given tissue may be influenced by the sex

of the roach. Thus, the enzymatic activity of the muscle and the hindgut in the male is significantly (beyond the .01 level) greater than that of the female. This sex difference in muscle is in accord with related findings of greater oxidative activity in male muscle as compared to that of the female (Barron and Tahmisian, '48; Sacktor and Bodenstein, '52; and Harvey and Beck, '53). The differences, due to sex, exhibited by the other tissues are not statistically significant. This contrasts the succino-cytochrome *c* reductase with the cytochrome *c* oxidase system, since it was observed (Sacktor and Bodenstein, '52) that the foregut, nerve cord and brain of the female had greater oxidase activity than did the male; and suggests that perhaps other cytochrome *c* reductase systems, such as diphosphopyridine (DPN)- or triphosphopyridine (TPN)- linked reductases are involved in the respiratory metabolism of these tissues.

The present data also enable a comparison, for each tissue, of the ratio between cytochrome *c* oxidase and succino-cytochrome *c* reductase activity. This is of interest since the oxidase is the only known enzyme complex in animal tissues involved in the biological oxidation of cytochrome *c,* whereas a number of reductases, including succino-reductase, are involved in the biological reduction of cytochrome *c*. With the use of previously reported data on cytochrome *c* oxidase (Sacktor and Bodenstein, '52), it was found that the oxidase: reductase ratio for the various tissues ranged from 4:1 to 16:1 (table 1). Under these conditions, muscle had 6 times as much oxidase as it had succino-reductase activity. This is in reasonable agreement with the data of Harvey and Beck ('53), who, by manometric techniques, found a ratio of 8:1 for this tissue. Thus, the roach data are in contrast with those obtained from isolated mitochondria of housefly flight muscle where only a 2:1 ratio was obtained (Sacktor, '53). In the roach the highest ratios were found in the brain and nerve cord, and this again suggests the important role of other cytochrome *c* reductases in the metabolic scheme in these tissues.

SUMMARY

The succino-cytochrome *c* reductase activity of various tissues of the American cockroach was investigated. It was found that these tissues differ in reductase activity and may be rated, in decreasing order, approximately as follows: muscle, hindgut, fat body, foregut, midgut, Malpighian tubes, brain and nerve cord. The enzymatic activity of the muscle and the hindgut of the male is significantly greater than that of the female. The cytochrome *c* oxidase:succino-cytochrome *c* reductase ratio for the various tissues ranges from 4:1 to 16:1. Muscle has a ratio of 6:1, whereas the highest ratios are found in the brain and nerve cord.

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RETINAL ACTION POTENTIAL — THEORY AND EXPERIMENTAL RESULTS FOR GRASSHOPPER EYES [1]

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THREE FIGURES

INTRODUCTION

When photoreceptors are illuminated a sequence of measurable changes occur which are instrumental in mediating the sense of vision. These changes are: (1) the absorption of radiant energy by a photolabile pigment or pigments and changes in the state(s) of the pigment (Granit, '47; Wald, '51); (2) a relatively long latent period following the onset of illumination during which no electrical changes are detectable; (3) a potential change measurable across the retina with extracellular electrodes called the retinal action potential (Hartline, Wagner and MacNichol, '52) which begins at the end of the latent period; (4) the initiation of nerve impulses in the axons of the sense cells (Hartline and Graham, '32; Granit, '47).

It has been frequently suggested (Hartline, '35; Wulff, '43; Granit, '47) that the retinal action potential is a generator potential producing local currents and initiating the trains of impulses in optic nerve axons. Recently it has been demonstrated (MacNichol, Wagner and Hartline, '53) with an intracellular electrode, that illumination causes a prolonged depolarization of the sense cells (presumably the eccentric sense cells) in the ommatidia of the Limulus lateral eye and that this depolarization is accompanied by the ap-

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy and Syracuse University, NR 119–266.

pearance of nerve impulse trains in the same cell. The depolarization measured with the intracellular electrode presumably is the intracellular sign of the retinal action potential recorded with extracellular electrodes. Although the retinal action potential has been shown to be directly associated with the nerve impulse discharge only in the case of the lateral eye of Limulus, we subscribe to the general idea that retinal action potentials are generator potentials.

Between the photochemical event in vision and the production of the retinal action potential processes and/or reactions must occur which produce the potential change. These coupling reactions between the photochemical event and subsequent events in vision have long received the attention of investigators (Hecht, '19; Hartline, '28; Jahn, '47; Wald, '51). Despite these efforts, the nature of the coupling processes remains obscure. It is the purpose of this paper to present a possible kinetic model of coupling processes which quantitatively describes many characteristics of the retinal action potential. It has been used in a predictive capacity to indicate fruitful new directions for experimental work.

Wulff and Pandazi ('51) reported that flash durations from 15 msec. to 1.0 second at constant intensity administered to a dark adapted photoreceptor produced electrical responses whose magnitude increased but whose latent periods were constant. These data thus indicated that two processes may be involved — one controlling the potential, the second controlling the latency. Two models were formulated, the first to describe the relation between intensity, flash duration and magnitude of the retinal action potential; and the second to describe the relation between intensity, flash duration and the latent period of the retinal action potential.

METHOD

The experiments were performed on dark adapted grasshoppers *(Melanoplus differentialis)*. The animals were securely fastened and a chamber was built about each of the

two lateral compound eyes. These chambers were filled with 0.9% NaCl solution, and each chamber made contact with a calomel half cell through a salt bridge. The half cells were connected to the input grids of a D.C. amplifier. The animal was so placed in a plastic container that the cornea of one eye could be exposed to a light flash admitted by the opening of a series of shutters, while the other eye remained in total darkness.

The interior of the chamber was ventilated by a constant stream of air at the temperature of water which circulated constantly through two copper heat exchangers built into the top and bottom of the animal chamber. Temperature was measured in terms of voltage developed by a copper-constant in thermocouple, with reference to a similar junction at 0°C, using a potentiometer circuit. Temperatures were regulated within $\pm$ 0.2°C of any given value. The air inside the chamber was approximately saturated with water vapor.

The animal chamber was securely fastened to a movable stage inside a Faraday cage, and the eye of the animal was oriented so that the cornea was at the focus of the light beam. The cage was then shielded from light and the animal was permitted to dark adapt.

Experiments were begun after two to 12 hours of dark adaptation. An interval of one hour between flashes proved to be necessary to permit complete recovery from the effects of preceding flashes. It was desirable to obtain a complete set of data from each animal; consequently the experiments were of several days duration. The grasshoppers, however, were not adversely affected by the experimental treatment and even after two weeks in the experimental chamber, resumed a normal existence upon return to the colony.

After the temperature had reached the desired level the experimental procedure consisted of adjusting the intensity of the light to the desired level, using Wratten neutral filters, and admitting a flash of light of 100 microseconds duration.[4]

[4] The high speed shutter was designed by Frank J. Fry and assembled under his supervision. We express our appreciation to him.

The electrical responses of the photoreceptor and of a photocell, to which a portion of the light beam had been diverted, were fed into D.C. amplifiers the outputs of which were recorded by pens on moving paper and by photographing the display on the face of a two-channel cathode ray tube. The paper receiving the ink tracing moved at 25.0 ± 0.25 cm per second, and the sensitized paper photographing the cathode ray tube display moved at 50.0 ± 0.25 cm per second. After each record the deflection produced by a pulse of known voltage was recorded. A perforated disk driven by a synchronous motor past a light source supplied the time base of the camera. After an adequate interval for dark adaption, another flash was admitted. Flash duration was varied by steps of 0.5 log units from 100 microseconds to 0.5 seconds. Intensity was varied by a factor of 10 from 10^{0} (unit intensity = 11,800 foot candles at the cornea) to 1×10^{-4}.

A total of 16 experiments were performed on grasshoppers. Although all the data obtained exhibited similar relations, single sets of data were selected for the text.

The measurements of magnitude and the latency were made as follows: (1) the base line was extended below the response and the point of greatest deflection was measured and the voltage computed; (2) the point where the tracing could be seen to leave the base line was marked as the end of the latent period and the interval from onset of illumination to this point was measured and converted to seconds. The experimental error has been estimated at ± 3% and the reproducibility of data from the same experimental animal was found to be about ± 10%.

A. Data and theory pertaining to retinal action potential magnitude

When the eye of a grasshopper is illuminated with a short flash of light there ensues a period of time during which no measurable electrical change occurs. This is followed by

a gradually developing negativity at the illuminated cornea which reaches a crest and then declines (fig. 1). If the flash duration is maintained constant and the intensity is decreased, a series of responses are obtained of decreasing magnitude and increasing latency. Similar series of responses have been obtained with a variety of flash durations.

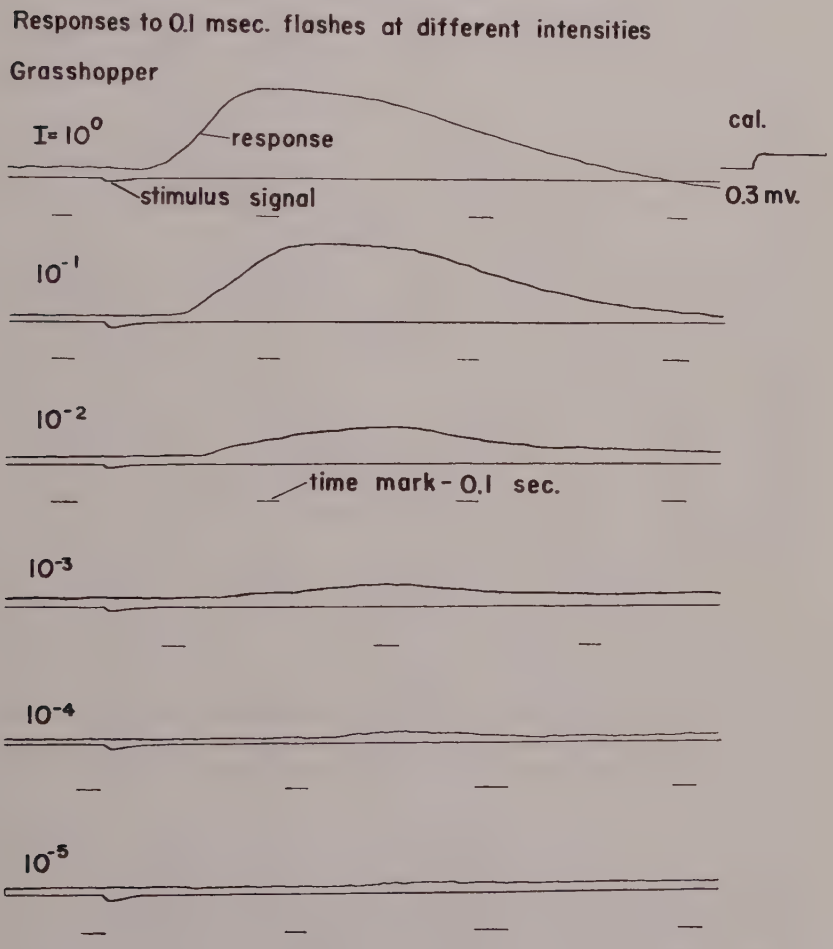

Fig. 1 A series of electrical responses recorded from the eye of a grasshopper *(Melanoplus differentialis)* illuminated with flashes of 0.1 millisecond duration and varying intensity. The recording paper speed for the grasshopper records was 50 cm per second. The records have been retouched.

1. The effect of intensity and duration of illumination on the retinal action potential magnitude. The data obtained from one grasshopper maintained in the dark at 30°C are presented in figure 2. The dots of figure 2 relate peak magnitude of the retinal action potential to the logarithm of the flash duration, measured in seconds, at 5 different intensities.

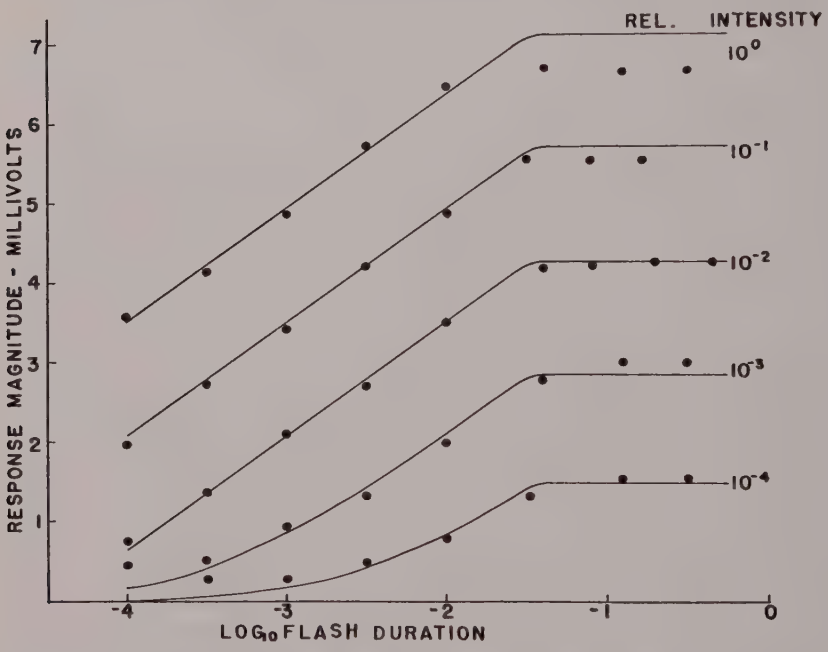

Fig. 2 The closed circles represent the maximum voltage recorded from grasshopper eyes in response to illumination with different intensities and exposures. The curves represent the theoretical relationship between voltage, intensity and flash duration given by expression (4) of the analysis.

The response magnitude at constant intensity increases with increasing flash duration and tends to reach a constant value at flash durations in the range 0.01 seconds to 0.025 seconds. (The specific value is determined by the intensity.) Similar data were obtained in 15 other experiments.

2. The kinetic model of a potential producing reaction. The model formulated to describe the magnitude characteristic

of the retinal action potential may be verbally stated as follows: (1) light acts on a photosensitive substance S in the photoreceptor producing a material C; (2) the substance C accumulates at a rate proportional to the light intensity and is depleted at a rate proportional to the difference between its concentration at any time and its concentration in the dark adapted, unilluminated eye; and (3) the potential magnitude developed after the lapse of the latent period is proportional to the logarithm of the concentration of C at the instant the flash ends.[5]

In the formal description of the essential characteristics of the model, capital letters designate substances and lower case letters designate concentrations and/or magnitudes. We assume that the exposure of a substance S to light produces a substance C (Wald, '51). Let the time rate of conversion of S be given as follows:

$$\frac{ds}{dt} = -aI, \tag{1}$$

which implies that the amount of S is not appreciably depleted during illumination. We assume that N molecules of C follow from the one of S and that the concentration of C changes in accordance with the following expression:

$$\frac{dc}{dt} = bI - k(c - c_1), \tag{2}$$

where b is proportional to $N \cdot a$ and k is the decay constant. If the concentration of C in the dark adapted unilluminated eye is c_1, and if the illumination begins at $t = 0$ and stops at $t = t_f$ then from (2) it follows that the concentration of C at a time equal to or greater than t_f is

$$c = c_1 + \frac{bI}{k}(1 - e^{-kt_f}). \tag{3}$$

We assume that the magnitude of the voltage difference, E, measured across the retina is proportional to the logarithm of the ratio of c to c_1. This is expressed as follows:

$$E = a \log \left[1 + \frac{bI}{c_1 k}(1 - e^{-kt_f})\right], \tag{4}$$

[5] The theory is being extended to include the depletion of the substance C after termination of the flash.

where E is the voltage, t_f is the flash duration and α is the proportionality constant.[6]

The continuous lines in figure 2 were computed using relation 4. The general agreement between data and theory is good. Two discrepancies deserve comment. First, the predicted plateau value for the response magnitude versus log t_f curve for intensity 10° (fig. 2) is higher than the measured plateau values. This discrepancy can be removed if the theory is modified to include a factor for depletion of S, the photosensitive material, during the course of illumination. The inclusion of this factor into the theory is readily achieved. It has the effect of decreasing plateau values at high intensities and also moves the bend in the response curves to the left.

Secondly, there is deviation between theory and data at the low intensity, short flash duration end of figure 2. We believe this discrepancy exists because the retinal action potential of the grasshopper eye in response to low levels of illumination is confused by a second potential wave whose characteristics are not amenable to experimental evaluation from our data (fig. 1).

B. Data and theory pertaining to the latent period

1. The effect of intensity and duration of illumination on the latency of the retinal action potential. The data obtained from one grasshopper maintained in the dark at 20°C are presented in figure 3. The data, represented by the circles and crosses, are the latent periods in seconds plotted as a function of the logarithm of the intensity for different flash

[6] The constants may readily be evaluated as follows: (1) the quantity α is equal to the slope of the linear portions of the curves of response magnitude versus log flash duration of figure 2, symbolically $\alpha = \frac{\Delta E}{\Delta \log t_f}$; (2) the rate constant, k, may be evaluated from the flash duration, t_{fo}, at the intersection of the linear rising portion and the plateau region for intermediate intensities, using the relation $k = 1/t_{fo}$; (3) the quantity $\frac{b}{c_1}$ can be determined from plateau values of the response magnitude for intermediate intensities by using the relation

$$E = \alpha \log \left(1 + \frac{bI}{c_1 k}\right).$$

durations. The latency exhibits a progressive increase as the intensity is decreased (see also figure 1). At any one intensity, the latency increases as the flash duration decreases. The change in latency when the flash duration is decreased from 0.1 second (flash duration greater than the latent period)

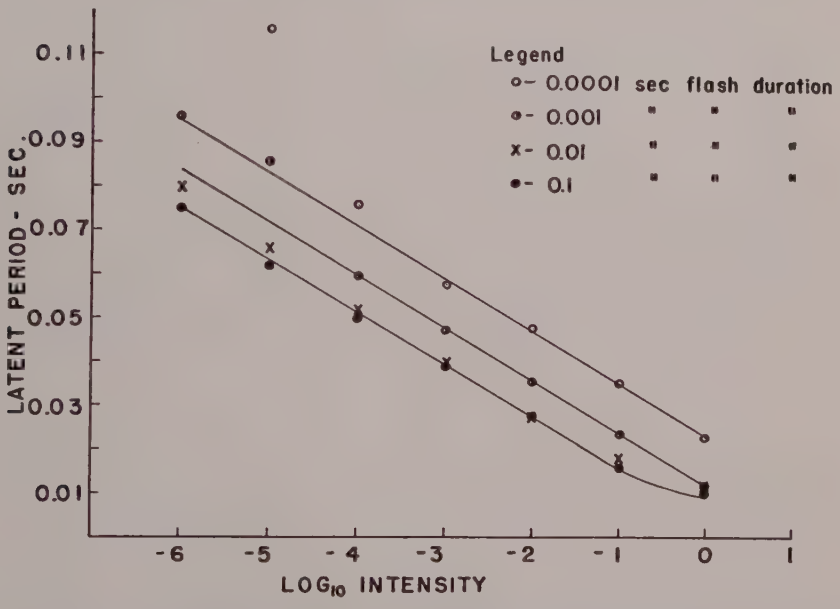

Fig. 3 The circles and crosses represent the latent period of the retinal action potential of the grasshopper plotted as a function of the logarithm of the intensity with the duration of exposure as a parameter. The curves are the theoretical relationship between latent period, intensity and flash duration given by expressions (6) and (7) of the analysis. The theoretical curve corresponding to the experimental points for 0.01 second flash duration (crosses) has been omitted because it is so near the curve for 0.1 second flash duration as to be almost indistinguishable on a graph the size of the figure.

to 0.01 second is very slight. For changes in flash duration from 0.01 second to 0.001 second and from 0.001 second to 0.0001 second the changes in latency are considerably greater. However, the percentage change in latency from exposures of 0.1 second to 0.0001 second is nevertheless very small compared to the thousand-fold change in the exposure.

2. The kinetic model of a process controlling the latent period. A model was formulated to describe the relation between the latent period of the retinal action potential and the intensity and duration of illumination. The model may be described as follows: (1) light, acting on a photosensitive substance S produces a substance or factor P; (2) P accumulates by two processes, one at a rate proportional to the intensity of illumination and the other proportional to the concentration of P. The second process is autocatalytic; (3) the substance or factor P accumulates and, when it reaches a critical concentration, p_c, the electrical response begins, that is, the latent period terminates.

In the formal description of the essential characteristics of the model capital letters designate substances or factors and lower case letters designate concentrations and/or magnitudes. The rate of production of P is given by the following relation:

$$\frac{dp}{dt} = hp + nI \qquad (5)$$

where p is the concentration or magnitude of the factor P. The constant h is the autocatalytic rate constant. If we assume that $t = 0$, $p = 0$ then (5) yields for the latent period, t_L, the following relation when the flash duration is equal to or less than the latent period ($t_f \leq t_L$)

$$ht_L = (1n\ 10) \log \left[\frac{hp_c}{n(1 - e^{ht_f})} \right] - (1n\ 10) \log I \qquad (6)$$

where t_f is the flash duration. When the flash duration is equal to or greater than the latent period ($t_f \geq t_L$) then (6) is replaced by

$$ht_L = (1n\ 10) \log \left[\frac{hp_c}{nI} + 1 \right]^{7} \qquad (7)$$

[7] The constants in these equations (6 and 7) are easily evaluated from the latency data, figure 3. The constant, h, can be evaluated from the slope, m, of the line drawn through the lowest set of points in figure 3, by using the relation $m = \frac{-1n\ 10}{h}$. The intercept, I_i, of the same line on the horizontal axis yields the constant $\frac{p_c}{n}$ from $I_i = \frac{hp_c}{n}$.

Although the theoretical development was stimulated by the observation that the latent period was not affected by flash duration from 0.015 second to 1.0 second (Wulff and Pandazi, '51), the theory predicted that latent period should vary with flash duration. The data presented in figure 3, which extend over a greater range of flash durations from 0.0001 second to 0.1 second, do indicate that the flash duration affects the latent period. The continuous lines in figure 3 are computed using equations (6) and (7).

It is noted from figure 3 that the experimental data for short flash durations and low intensities deviate from the linear relation indicated by the theory. This deviation is probably associated with the difficulty of measuring the latency from the tape records at low response magnitudes. As the magnitude of the response becomes small the error introduced in determining the position on the record at which a deviation from the base line occurs becomes larger and tends to yield greater values of the time. This explanation of the discrepancy between theory and data was substantiated by observing that when the gain of the amplifier system was reduced (resulting in smaller deflections on the tape) the deviation from linearity occurred at high intensities and larger flash durations.

The deviation from linearity at high intensities and long flash durations, figure 3, can be satisfactorily accounted for by the appreciable depletion of a light sensitive substance. This modification of the theory has been formulated and applied to the data (lowest curve of figure 3).

DISCUSSION

The evidence which has accumulated bearing upon the peripheral visual process in photoreceptors suggests that the initial event in vision is the absorption of radiant energy by light sensitive unstable pigments, of which rhodopsin is the most familiar example. Following this photochemical event there occur, after the lapse of some time, electrical changes in the photosensitive cells which culminate in the

discharge of nerve impulses. Recently MacNichol et al. ('53) demonstrated that illumination of the lateral eye of Limulus produces a depolarization of sense cells and that the discharge of nerve impulses is associated with the wave of depolarization. One of the major problems in the peripheral visual process which awaits solution is an elucidation of the events which follow the photochemical event and precede the electrical events.

The work of Wald and co-workers which has centered around the photochemistry of rhodopsin, the vertebrate rod pigment, has provided some data which may prove to be important in the coupling processes between the photochemical and electrical events. Wald ('51) has identified two substances, lumi-rhodopsin and meta-rhodopsin, which have a brief existence at temperatures compatible with life. The transient existence of these substances suggests that they may play a role in peripheral vision. More recently Wald and Brown ('52) have demonstrated that rhodopsin, illuminated when in an amperometric titration cell, binds silver ions. Various lines of evidence led Wald to suggest that the observed change in cation concentration was caused by sulfhydryl groups, suddenly uncoupled by the action of light, absorbing cations in the solution being titrated. The time characteristics of the current change were not indicated. An event of this kind, producing an electrical change, might be identified with the model pertaining to the potential magnitude.

The kinetic model presented above consists of two parts, the potential magnitude process and the latency process. The two processes are both initiated by light but, subsequently, there is no obvious relation between the two. It may, indeed, be debated whether two separate processes are necessary to account for the characteristics of the retinal action potential. Our belief that two processes are necessary is based upon the following observations. (1) The starting point for the development of the theory was the difference in the behavior of potential magnitude and latent period with respect to the

duration of illumination. This difference in behavior is further emphasized by the results obtained in the course of experiments reported above. The magnitude versus log t_f data (fig. 2) indicate there is at each intensity a flash duration beyond which the response magnitude will not increase, regardless of the length of flash. This flash duration, called the critical duration (Hartline, '28), does not correspond to the latent period. Our experiments show that there is no fixed relationship between the critical duration and the latent period which holds for all the animals examined. (2) Further, the models have been used to predict results which have been strikingly verified by experiment. The latency model accurately predicted the dependence of latent period on flash duration at constant intensity which was later determined experimentally (fig. 3). (3) The effect of temperature on the characteristics of the electrical response of the grasshopper eye again points to two distinct processes (Fry, Wulff and Brust, '55). In the grasshopper temperature does not affect the response magnitude on the rising portions of the magnitude vs. log t_f curves but the latencies for the same responses are markedly temperature dependent. Our conviction of the necessity of two coupling processes has been considerably strengthened by the temperature effect on the latency and magnitude of the retinal response.

Another prediction made by the theory concerns the effect of temperature on the plateau magnitudes of the response curves. Expression (2) of the potential magnitude model suggests that, if the decay process is temperature dependent, then a rise in temperature should accelerate the decay process and decrease the plateau magnitude. Conversely, a drop in temperature should slow the decay process and increase the plateau magnitude. These predictions have been confirmed experimentally and are presented in the following paper.

The models have thus far stimulated considerable experimentation. In addition, they suggest that the time course of the coupling processes are sufficiently slow to be measurable spectrophotometrically, if the optical densities of materials

are great enough. Experiments are underway to test this idea.

It is probable that the elucidation of the potential generating mechanism in photosensitive cells may increase our insight into generator potential phenomena within the nervous system.

SUMMARY

1. The magnitude of the retinal action potential obtained from dark adapted eyes of grasshoppers is a function of the intensity and duration of illumination. The form of this relation is consistent from one animal to another.

(a) The characteristics of the relation between action potential and intensity and duration of illumination are reasonably accurately described by a theory which assumes that:

(1) The light acts on a photosensitive substance S producing a material C whose concentration manifests itself as an emf across the retina after the lapse of a latent period.

(2) The time rate of production of C is proportional to the light intensity and the rate of depletion, during illumination, is proportional to the difference between the concentration of C at any time and the equilibrium concentration in the dark adapted eye.

(3) The maximum values of the emf generated after the lapse of the latent period are proportional to the logarithm of the concentration of C at the instant the flash ends.

(b) The experimentally determined relation between response magnitude and logarithm of flash duration, for any single intensity, exhibits the following: (i) a slowly rising phase at short flash durations; (ii) a linear region at intermediate flash durations; and (iii) a plateau region as the flash duration increases further.

(1) In the linear region and below, curves for different intensities show that a constant value of response magnitude is obtained for a fixed value of the product of intensity and flash duration. This characteristic is derivable from the hypothesis that C is generated at a rate proportional to the intensity of illumination and that the emf across the retina is proportional to the logarithm of the concentration.

(2) The magnitudes of the response in the plateau region (i.e., long flash durations) are, for equal logarithmic increments of intensity, uniformly spaced at intermediate response magnitudes, compressed together at low response magnitudes and also compressed together at high response magnitudes. The existence of the plateau regions is correlated with the depletion process for substance C. The data are quantitatively described by the theory presented in this paper except for the compression at high response magnitudes. To explain this compression it is sufficient to include in the theory the dependence of the rate of the initial photochemical process on the concentration of the light sensitive substance S, which for high intensities and long flash durations, is appreciably depleted during the flash.

2. The latency of the retinal action potential is, for a single flash duration, a linear function of the logarithm of the intensity over almost the entire range of intensities used in the experiments. The latency is a relatively insensitive function of the flash duration.

(a) The characteristics of the relation between latency and intensity and flash duration are accurately described by a theory which assumes that:

(1) The time rate of production of a factor or state P whose magnitude determines when the elec-

trical response begins is proportional to the intensity of illumination and is also autocatalytic.

(2) When the magnitude of the factor P reaches some critical value the electrical response begins.

(b) (1) The linear portion of the experimentally determined relation between latent period and the logarithm of intensity for a constant flash duration is accurately described by the theory based on an autocatalytic rate process initiated by the light.

(2) The latent period varies only very slightly with flash duration as the flash duration becomes less than the latent period. As the flash duration further decreases the latent period changes more rapidly but still quite slowly compared to the changes in flash time. This characteristic of the latency is quantitatively described by the theory just mentioned.

(3) The experimental data show, for the highest intensities and longest flash durations, that the latent period is no longer a linear function of the logarithm of the intensity. As the intensity increases the latency appears to approach a finite non zero value. This aspect of the latent period is explained by modifying the theory to take account of the appreciable depletion of a light sensitive substance which occurs at high intensities and long flash durations.

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RETINAL ACTION POTENTIAL — EFFECT OF TEMPERATURE ON MAGNITUDE AND LATENCY IN THE GRASSHOPPER [1]

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TWO FIGURES

INTRODUCTION

The investigation of the effect of temperature on the magnitude and latent period of the retinal action potential elicited by illuminating the compound eye of grasshoppers was undertaken for three reasons: (1) to provide additional evidence in support of the contention that the potential magnitude and latent period are controlled by different processes; (2) to evaluate the effect of temperature on some of the constants in the kinetic model; and (3) to determine the validity of certain predictions suggested by the kinetic model developed in the preceding paper (Wulff, Fry, Linde, '55).

The model for the potential generating mechanism postulates that the action of light on a photosensitive material produces a material designated by C which manifests itself, after the lapse of the latent period, as an e.m.f., the response magnitude of the retinal action potential. The material C accumulates at a rate proportional to the intensity of illumination and it is depleted, during illumination, at a rate proportional to the difference in its concentration at any time and the concentration in the dark adapted eye. This idea is formally represented by equation 2, page 253. If the depletion reaction is thermolabile and the accumulation reaction is thermostable

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Syracuse University, NR 119-266.

then a reduction in temperature should increase the plateau magnitude of the potential versus log t_f (t_f — flash duration) curve but should have negligible effect on the rising phase of the same curve if the assumption is made that the equilibrium concentration of C does not vary much over the temperature range 10°–30°C. The experimental results summarized herein show that the response magnitude versus log t_f relation has this characteristic for all but the highest intensities.

The kinetic model controlling the latent period postulates that light acting on a photosensitive substance produces a state or factor P. The rate at which P accumulates is the sum of two rates, one autocatalytic and one proportional to the light intensity. It is assumed that the latent period terminates when the magnitude of P reaches some critical value. These ideas are formally presented in equations 5, 6 and 7, page 256. The model indicates that increasing temperature should produce shorter latent periods and, in particular, that the slope (absolute value) of the linear portion of the curves of latent period versus logarithm of intensity should be greater the lower the temperature. In addition the theory also indicates that the extended linear portions of these curves should intercept the log I (I — intensity) axis at values such that the critical value of P is lower at higher temperatures. The results of temperature studies on grasshopper eyes (Jahn and Crescitelli, '39, Jahn and Wulff, '42) and those on other photoreceptors (Hecht, '19, Nikiforowsky, '11) show that the duration of the latent period is inversely related to the temperature. The experimental data presented herein verify the two specific predictions indicated by the theory of the latent period regarding the changes with temperature.

MATERIALS

Grasshoppers *(Melanoplus differentialis)* used in these experiments were prepared in a manner described previously (Wulff, et al., '55). In these experiments the temperature of the experimental chamber was held at 10°, 20° and 30°

for periods of days to permit the collection of adequate data. The experimental animals seemed not to be adversely affected by the experimental procedure. In all other respects the methods employed were identical to those already described (loc. cit.). Temperature experiments were performed on 15 animals.

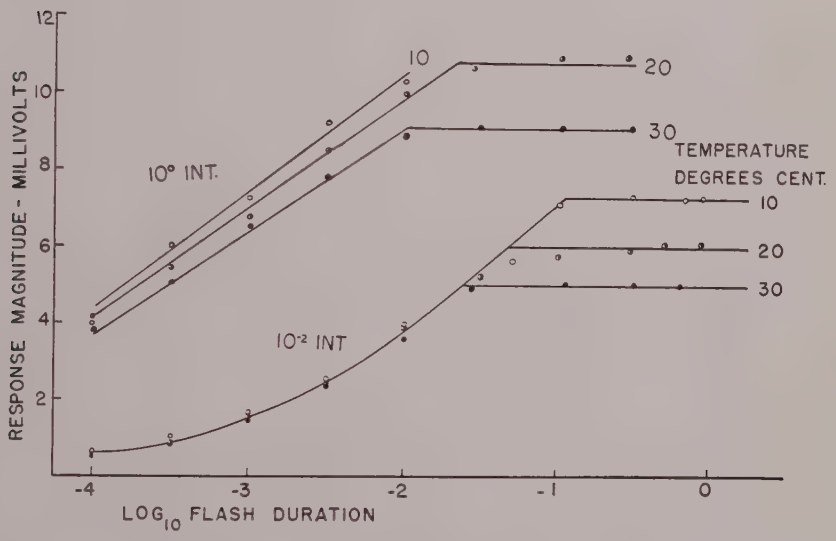

Fig. 1 Magnitude of the grasshopper retinal action potential plotted as a function of the logarithm of the flash duration, for two intensities of illumination and three temperatures. Unit intensity represents 11,800 foot candles at the cornea.

RESULTS

A. The effect of temperature on the potential magnitude. The relation between temperature, duration, and intensity of illumination is presented in figure 1. The data in the lower set of curves were obtained with an illuminating intensity of 118 fc. at the cornea and at three different temperatures. The data in the upper set of curves were obtained with an illuminating intensity of 11,800 fc. on the cornea. Similar results were found in 14 other temperature experiments on grasshoppers.

B. The effect of temperature on the latent period. The relation between temperature, intensity of illumination and latent period is given in figure 2. An intensity value of unity on the scale of figure 2 corresponds to 11,800 fc. The lowest set of points was obtained upon illumination at given intensities for 0.05 secs. duration at 30°C. The upper set of points was obtained under the same conditions of illumination but at 10°C. The middle set of data was obtained upon

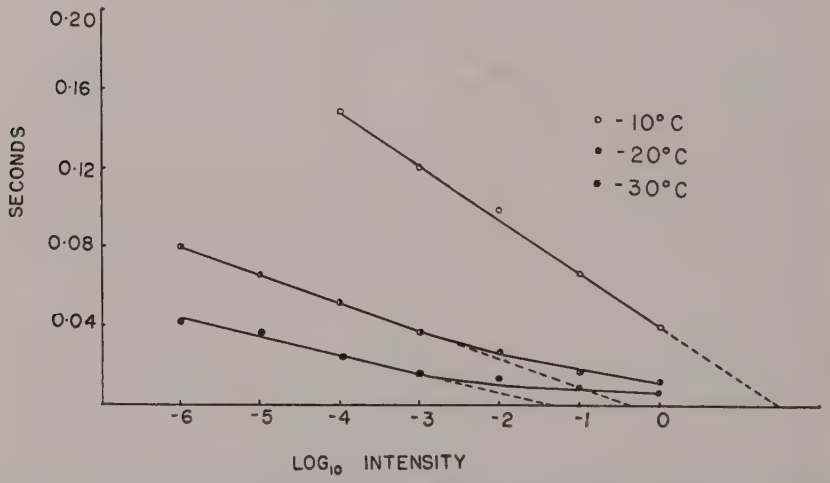

Fig. 2 The latent period of the grasshopper retinal action potential plotted as a function of the logarithm of the intensity for three temperatures. The 10°C. and 30°C. data were obtained from one animal using exposures of 0.05 secs. The 20°C. data were obtained from a different animal using exposures of 0.01 secs. Unit intensity represents 11,800 foot candles at the cornea.

illumination at given intensities for 0.01 sec. duration at 20°C. Similar results were found in 4 experiments of this type on grasshoppers.

C. The ratio of constants at different temperatures. For intermediate intensities (the lower curves of fig. 1) the constant, k, of the depletion reaction in the kinetic model (loc. cit.) is simply related to the reciprocal of the flash duration at the inflection point of the magnitude vs. log t_f curve; i.e. $k = \frac{1}{t_f}$. The temperature coefficient of k expressed as a ratio

of its values at two temperatures 10°C apart varies between 1.9 and 2.4 with an average of 2.2.

The rate constant, h, of the autocatalytic reaction in the latent period process (loc. cit.) can be evaluated from the slope, m, of the experimental curves in figure 2, as follows:

$$m = \frac{-\ln(10)}{h}.$$

The temperature coefficient of h expressed as a ratio of its values at two temperatures 10°C apart, and determined from the data of figure 2 is 1.7. This value is obtained using only the 10°C and 30°C curves of figure 2. The average temperature coefficient of h from 3 experiments is *1.50* with a range of *1.25* to *1.70*.

The constant p_c/n, which appears in the model for the latency process (loc. cit.), is evaluated from the intercepts of the linear portions of the curves in figure 2 on the log I axis by using the relation

$$I_1 = \frac{hp_c}{n}.$$

Numerically p_c/n is 3.84 $(10)^{-1}$ at 10°C, 2.46 $(10)^{-3}$ at 20°C and 1.64 $(10)^{-4}$ at 30°C.

DISCUSSION

The summary of experimental results, presented herein, concerning the changes in the magnitude and the latency of the retinal action potential of the grasshopper as a function of temperature constitute further support for the kinetic model (Wulff, Fry and Linde, '55) dealing with these characteristics at a single temperature.

The predictions of this model concerning the changes in latency with temperature are verified. The rate constant, h, of the autocatalytic process in the latent period mechanism should increase as the temperature rises. It follows from the theory that h is inversely proportional to the absolute value of the slope of the linear portion of the curves of latent period versus logarithm of intensity. The experimental data

(fig. 2) show that the slope increases as the temperature decreases. Thus, the prediction concerning the variation of the rate constant, h, as a function of temperature is verified. It is of interest to note that the value of the temperature coefficient of h (average *1.5*) is low for a chemical reaction. It is, of course, possible that the model for the latent period does not imply a chemical process.

The model also indicates that the intercepts of the linear portions of the curves of latent period versus logarithm of intensity on the log I axis should satisfy a specific inequality relation. That is, the intercepts should have values such that the magnitude of the factor P, derived from the experimental data, which determines the time of initiation of an electrical response, decreases as the temperatures increases. Since the parameter n is the rate constant for a light initiated process it would not be expected to vary much over the temperature range used in these experiments. The variation in the quantity p_c/n with temperature is then essentially a measure of the variation of p_c with the temperature. The numerical values for p_c/n given above, which are derived from the intercept values of the experimental curves, then verify the prediction of the model. The quantity p_c decreases rapidly as the temperature increases, between 10°C and 20°C it decreases by a factor of 156 and between 20°C and 30°C it decreases by a factor of 15.

The variation in the response magnitude at all but the highest intensities exhibits a striking behavior, which is consistent with the model. At a constant intensity, response magnitude as a function of the flash duration is expressed as a rising curve independent of the temperature until it increases to a value which, for further increases in flash duration, becomes constant. This constant value is temperature dependent.

It was predicted on the basis of the model that the plateau value should certainly vary with the temperature since the

rate constant, k, of the chemical reaction determining the rate of depletion of C determines the value of the flash duration at which the plateau starts. The temperature coefficient of k (average $Q_{10} = 2.2$) calculated from the experimental data is within the usual range of values characteristic of many chemical reactions.

It is not possible to conclude theoretically that the rising phase of the relation between response magnitude and flash duration should be independent of the temperature; actually a variation with temperature is not opposed by the model. However, the nonvariation of this rising phase with temperature constitutes strong support for the model since this particular characteristic is realized by assuming that the equilibrium concentration of C in the dark adapted eye is practically constant over the temperature range of interest in this paper.[4]

Such an assumption is not unreasonable but it is not implied that because this situation obtains in grasshopper eyes that this would be characteristic of eyes in general.[5]

The deviation from the model which occurs at the highest intensities is exhibited as a compression of the plateau values and change in slope of the linear rising phase of the relation between response magnitude and logarithm of flash duration. This sort of deviation at the highest intensities and constant temperature appears to be at least partially associated with the appreciable depletion of the light sensitive substance during the time of the flash (Wulff, Fry and Linde, '55).

[4]The constants b and α are also involved in determining the form of this relation. However, b is the rate constant of the light initiated process and consequently should not vary much over the temperature range of the data presented here. The constant α which is the proportionality parameter relating emf to logarithm of concentration would not be expected to vary much over this same temperature range if it is assumed that this parameter has a temperature coefficient similar to that characterizing many voltaic processes.

[5] The effect of temperature on the characteristics of the retinal action potential of the lateral eye of Limulus is being investigated. The results indicate that the effect of temperature on response magnitude of the Limulus retinal action potential is different from that of the grasshopper but the effect on latency is similar.

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THE EFFECT OF POTASSIUM CHLORIDE ON THE EXCITABILITY AND CONDUCTION OF THE LOBSTER SINGLE NERVE FIBER

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THIRTEEN FIGURES

INTRODUCTION

Large crustacean nerve fibers have higher rheobase, shorter chronaxie time or larger k (Blair, '32a, b) values than small nerve fibers (Easton, '52; Wright, Adelman and Coleman, '53; Wright and Coleman, '54), and are so highly accommodating that some, fast closers, respond with but one impulse even to strong DC stimulation. Small fibers, particularly openers, in the same nerve trunk are almost totally non-accommodating and respond with long trains of impulses to the same DC stimulation (Wright and Adelman, '54). The slow closer fibers which are smaller than the openers, accommodate more than opener fibers, but far less than fast closers. A possible explanation for these differences is to be found in the excitability changes which have been observed to occur in a fiber continuously stimulated or simply allowed to deteriorate with time after isolation. Large fast closer fibers are fatigued more easily and cease conducting sooner after dissection than the smaller fibers. Small fibers such as openers and slow closers which are non-accommodating and respond repetitively when first prepared, become highly accommodating when continually stimulated or after long

periods of time and respond non-repetitively long before conduction fails completely.

Recently the magnificent work of Hodgkin, Huxley ('52) has established by voltage-clamp and radioactive tracer techniques that potassium leaks out of excised nerve fibers rapidly and the rate of outflux is substantially increased by stimulation.

Excess potassium, although it may cause an initial lowering of threshold, has long been known to depress nerve excitability and produce conduction block (Blumenfeldt, '25; Rosenberg, '30; Graham, '33; Bouman, '37; Lehman, '37; Carleton, Blair and Latchford, '38; Graham and Blair, '47; and Grundfest, Shanes and Freygang, '53). Furthermore, local highly damped oscillations, which have been recorded with subthreshold stimulation (Cole, '50) or following the propagated spike (Shanes, '49) from the squid giant axon, are depressed and damping is increased by excess potassium (Shanes, '49). The damping also increases with time and the oscillations slowly disappear from the response of this giant fiber. And experiments by Solandt ('36) and Parrack ('40) have indicated that potassium increases accommodation in frog nerve.

These observations have led to the suggestion (Wright and Adelman, '54) that potassium leaking from the fiber interior to the thin film of saline surrounding the fiber, when raised in oil, raises the outside concentration enough to depress the excitability of the fiber. If this suggestion is correct, it should be possible to transform the excitability constants of the smaller fibers, slow closers or openers, to those of the large fibers like a fast closer or medial giant by simply adding potassium to the normal medium.

To see if an opener fiber can be made to respond like a fast closer by merely increasing the potassium concentration in the physiological solution, a series of experiments designed to test the effects of different concentrations of potassium chloride in sea water on the single non-myelinated nerve fiber of the lobster have been carried out.

METHODS

Obviously to investigate the effect of a test solution upon a single fiber, it is most desirable to stimulate and record from regions of this fiber in the solution. The intracellular electrode technique, though tempting, is not feasible in this situation because low impedance electrodes are an absolute necessity in the study of excitability loss and the small diameter, only 25–60 μ, of the nerve fibers prohibits the insertion

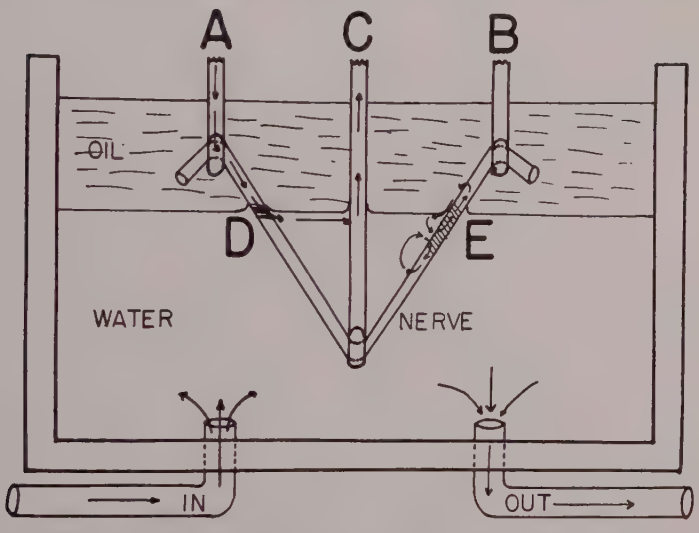

Fig. 1 The method of stimulating and recording from a single fiber partly in oil and partly in sea water or test solution. A — anode, B — recording electrode, C — cathode (ground), D — oil-water interface where excitation occurs, E — oil-water interface where spike (artifact) is recorded. See text.

of a large enough electrode to meet this requirement (Marmont, '49). Therefore, a technique was devised by which the test solution itself or rather the surface edge of it acts as both the stimulating and recording external electrodes. The new method is illustrated in figure 1.

A 1.5–2 cm length of nerve fiber is isolated in the meropodite section of the leg which is immersed in sea water in the small lucite compartment (fig. 1) fastened on the microscope stand. The fiber is cut away from the nerve bundle leaving tufts of

whole nerve at each end for handling purposes. After removal of the limb from the chamber, the fiber remains in the sea water, and the three platinized-platinum wire electrodes are lowered into the water on micromanipulators. The tuft on one end of the fiber is carefully lifted onto electrode A (fig. 1) and then the tuft on the other end of the fiber is "speared" by a fine needle with a wooden handle and the fiber threaded under electrode C and then placed over electrode B. The slack in the fiber is taken up gently by slight micromanipulation. Mineral oil, about 6 or 7 mm deep, is floated on the sea water and the level of the oil-water interface is adjusted (see below) so that A and B are in oil while C is in sea water (fig. 1). The stimulating current enters the fiber at A and leaves the fiber at D. The sea water surface acts as a "ring cathode" about the fiber and excitation will take place in this region (D). An artifact identical to the action potential and hereafter considered the action potential is recorded at E, as the action potential is conducted across the oil-water interface at this point, because the very abrupt conductance change of the external media is located between the recording leads C and B (Marmont, '40, p. 399). If the nerve fiber conducts over electrode B, a diphasic response is recorded, otherwise the spike appears monophasic. The sea water may easily be changed to test solution with or without varying the oil-water interface level by adjustment of stopcocks connecting with both inflow and outflow tubes. Excitation, conduction, and action potential all can be studied simultaneously while the test solution is affecting the fiber in the very region of the excitation and conduction. To prove that excitation and recording are taking place at interfaces D and E, it is only necessary to mount a fiber with a blocked area at a known region on the fiber and place this area between electrodes A and C or between B and C. Many fibers in the process of isolation are kinked or stretched slightly and a block develops at this point which can be accurately located by testing conduction along the fiber before it is cut from the nerve. As long as the kink is between A and D or between B and E, the

usual action potential recordings are obtained with the exception that diphasic responses are not observed. If the kink is anywhere between D and C or E and C no action potential is recorded. The spike can be "recovered" if the interface level is lowered below the block.

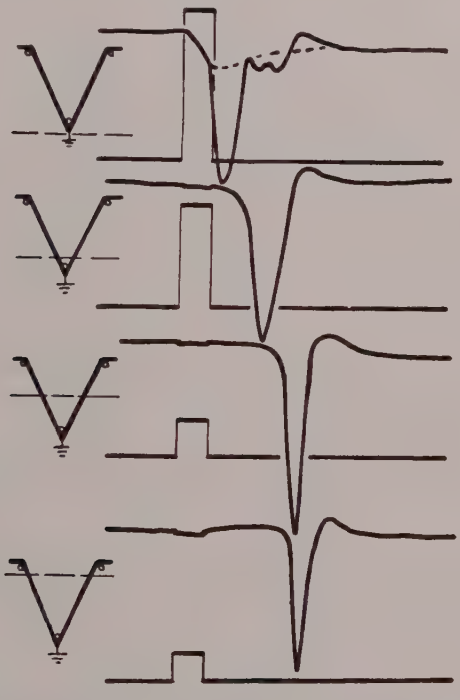

Fig. 2a Action potential and stiumlus recordings on dual sweeps from a single fiber mounted according to the method of figure 1 with oil-water interface at different levels indicated by dashed lines in diagram on left. See text.

A test of the method is illustrated in figure 2a. The recordings may be compared with records obtained using the Hodgkin technique, shown in figure 2b (Hodgkin, '39; Hodgkin and Huxley, '46), in which two pairs of electrodes for stimulating and recording are in oil and a non-conductive glass hook holds the middle portion of the nerve down in the sea water.

The position of the fiber and electrodes relative to the oil-water interface is sketched diagrammatically on the left in each figure. The action potential and the stimulus voltage used are shown to the right of the diagram of the position for that particular recording. The stimulus is just threshold

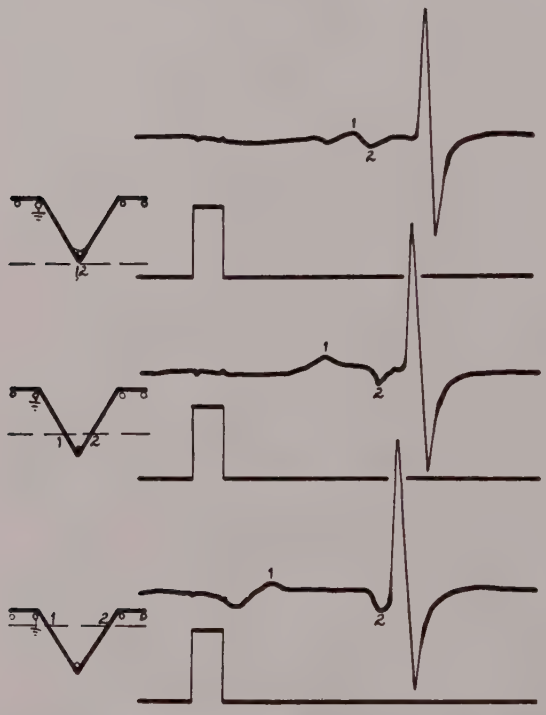

Fig. 2b Action potential and stimulus recordings on dual sweeps from a single fiber mounted across an oil-water interface in the Hodgkin "dip" method with the interface at different levels indicated by the dashed line in the diagrams on the left. See text.

voltage and 1 ms in duration. The action potential and stimulus are recorded on separate beams of a twin-beam oscilloscope (Dumont 322). No preamplification is necessary.

There are two very obvious differences between the recordings in figure 2a and the recordings in figure 2b. With both stimulating electrodes in oil the threshold voltage remains

constant while the spike latency (i.e., 1/velocity of conduction) increases as the interface level is lowered increasing the length of fiber in oil (fig. 2b, bottom to top). Under the same conditions by the new method, the threshold increases and the latency decreases (fig. 2a, bottom to top). The increase in threshold is due to the increase in distance and hence resistance (internal resistance of the fiber) [1] between the stimulating electrodes A and D (fig. 1) when the interface level is lowered. The increase in latency using Hodgkin's method (fig. 2b) as the interface is lowered is due to the decrease in conduction velocity in the low conductance oil medium. The decrease in latency with the new method (fig. 2a) as the interface is lowered is due to the decrease in conduction distance between the stimulating point D and the recording point E. This latency decrease is also visible in figure 2b between two small artifacts 1 and 2 produced by the exceptionally large spike as it crossed each interface also marked 1 and 2. These artifacts approach one another as the interface is lowered because the distance between their points of origin is being reduced. Normally these artifacts are not recorded with electrodes in this position because both electrodes are affected simultaneously instead of differentially as the spike crosses an interface and deflection is balanced out. When the fiber is totally in oil (top fig. 2a), stimulation and action potential recordings are taking place in the small cathodal area by the new method and hence the local response appears at the foot of the spike (dashed line) (Hodgkin, '37). Good nerve fiber preparations which have low thresholds, 15–30 mv, and conduct along the entire isolated length at the start of an experiment, maintain normal

[1] The parallel resistance, m, calculated from the data of Hodgkin and Rushton (1946, The Electrical Constants of a Crustacean Nerve Fiber. Proc. Roy. Soc. B., *133:* 444) for a 40 μ fiber is about 800,000 ohms per centimeter. The interelectrode distance was varied between 2 mm and 10 mm, and since the relationship between resistance and interelectrode distance is a linear one, a 5-fold variation in threshold would be expected for such an interelectrode distance change as shown in figure 2 a. The action potential amplitude was distorted by a loss of less than 10% by placing this fiber resistance across the 4 megohm input of the oscilloscope and no preamplification was necessary.

properties of excitation and conduction for 5–6 hours after placement on the electrodes in oil and sea water.

After the control data are taken in sea water, the test solution is allowed to pass through the chamber for 15 to 30 seconds, then the first test solution recordings are made.

Altogether 47 single nerve fibers were exposed to different concentrations of potassium chloride in sea water. These included 13 fast closers, 15 slow closers and 17 openers isolated from the lobster walking limb, and two giant fibers, one medial and one lateral dissected from the eosophageal commissure.

Stimulation and recording equipment was described previously (Wright and Coleman, Wright and Adelman, loc. cit.).

Sea water from Woods Hole was used as normal physiological solution. This contains about 0.7 gm potassium chloride per liter (Prosser, '51). Five times excess potassium chloride concentrated sea water was made up by adding 3 gm crystalline potassium chloride to one liter of sea water. This solution was diluted by adding sea water to any desired excess concentration of potassium chloride less than 5 times normal. Since the addition of 3 gm of potassium chloride to sea water made the solution slightly hypertonic (0.3%), the effect of almost double this hypertonicity produced by adding 20 gm of dextrose to sea water was studied as a control measure. The excitability and conduction properties of two single fibers were unchanged by this hypertonic sugar solution after two and three hours, respectively. The temperature of the sea water and test solution was held between 19° and 21°C. during all experiments.

Artificial sea water was made up with and without potassium chloride for potassium free solution experiments. These solutions were carefully buffered to pH 7.8, the same as the Woods Hole sea water.

RESULTS

When a highly excitable and non-accommodating fiber is subjected to excess potassium chloride in sea water, two very

obvious changes occur immediately: (1) a remarkably abrupt arrest of repetitive firing even with strong DC stimulation, and (2) a significant decrease in latency of response at rheobase stimulation. A typical series of records obtained from an excellent opener fiber preparation and shown in figure 3a

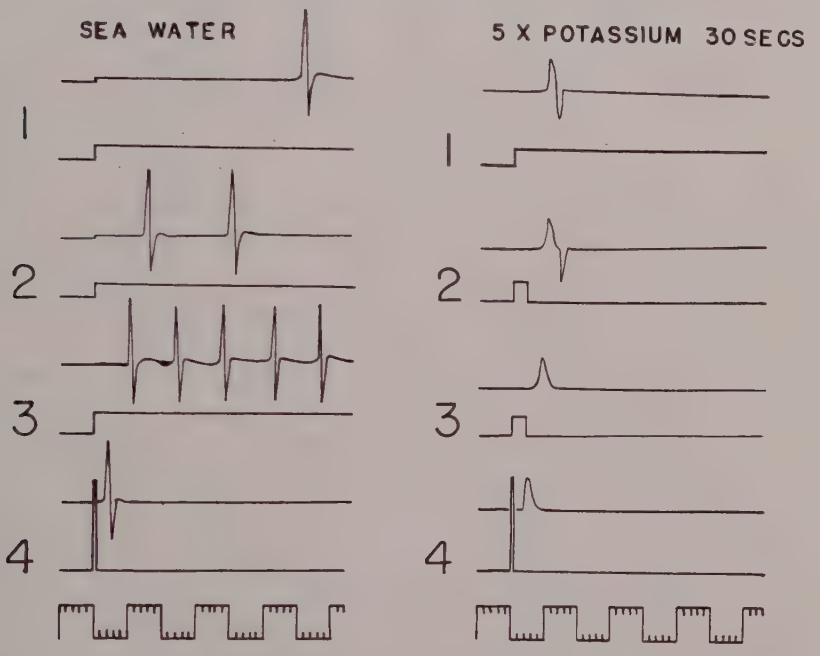

Fig. 3a Action potential from an opener fiber excited by rheobasic and stronger DC stimuli, 1–3, and a supramaximal 1 ms pulse, 4, on left in sea water. Recordings from the same fiber after 30 seconds to two minutes in sea water containing 5 × normal KCl on right. Rheobase stimulation, 1, 2 ms threshold shocks, 2–3, note reduced conduction velocity and block of second phase. Stimulation by 1 ms supramaximal shock, 4. See text. 20 mv. 5 ms calibration.

illustrate these changes. In sea water with rheobase stimulation, 23 mv (left column, picture 1), the fiber fired one diphasic impulse of 64 mv amplitude after a latency of 28 ms. With increasing stimulus strength the time to the first impulse was decreased to 5 ms and the fiber fired repetitively at a frequency of 60–150 spikes per second (pictures 2 and 3, left column). A 1 ms duration super-maximal shock reduced the

latency to a minimum 1.6 ms (picture 4, left column). The calculated velocity of conduction was 5 m/sec. This fiber was then totally immersed in 5 × potassium chloride sea water for 30 seconds and the oil-water interface level brought back to the original level, and the recordings in the right-hand column were made in the following two minutes. The rheobase had risen to 30 mv, the maximum latency time was only 5 ms and the action potential had decreased to 30 mv in amplitude (picture 1). Conduction slowed and then blocked between the recording interface and the recording lead, changing the diphasic spike to a monophasic one (pictures 2 and 3). The reason for a 2 ms stimulation shock instead of long duration DC in these figures is that strength duration curve data were being taken when this blocking was noticed and photographs were hastily obtained without taking time to reset the shock duration on the stimulator which happened to be giving 2 ms shocks at this time. The latency with supermaximal 1 ms shocks was exactly the same as in sea water (picture 4); in other words the velocity of conduction between the two oil-water interfaces had not changed. The rising phase of the spike was less steep so that the latency to the spike peak in the potassium solution was about 20% longer than in normal sea water. Similar results were obtained when opener fibers were subjected to 2.5 × normal potassium chloride concentration.

This rapid and reversible change from repetitive to nonrepetitive firing with DC stimulation always occurred with higher than normal concentration of KCl. The most obvious reason for such a change was a substantial increase in the accommodation of a fiber. Accommodation was tested by inserting a high resistance and variable capacitance in the stimulator output to "blunt" the square wave into an exponentially rising one (Solandt, '36). Typical experimental records from a fast and slow closer, double fiber preparation, are shown in figure 3b.

The upper row of pictures, 1–5, show the slow closer action potential and stimulus on the two sweeps while the fiber was

in sea water, 1–3, and after 5 minutes in 2.5 × KCl. Picture 1 shows the initial 35 mv spike elicited with 12 mv rheobase stimulation which is a bit blunted by the high resistance put in the circuit and the small capacity of the medium and preparation. With a 0.05 μf capacity in the circuit the rheobase is only raised slightly to 15 mv, although the slope of the exponentially rising voltage is very shallow, and the time con-

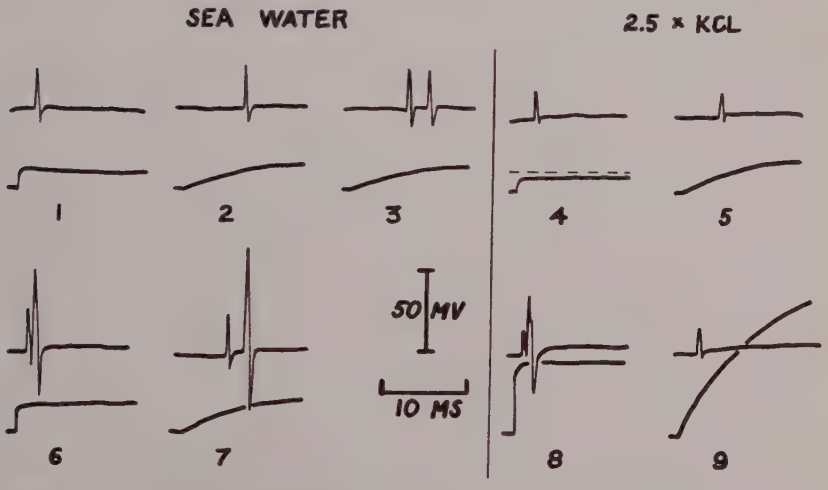

Fig. 3b The effect of 2.5 × KCl in sea water on a double fiber fast and slow closer preparation. The action potential of the slow closer excited by DC (1) and blunted shocks (2 and 3) in sea water and in excess KCl (4, 5). Note the reduced threshold in 4, the normal one is indicated by dashed line. With stronger stimulation the response of both fibers is recorded (6, 7, 8) but fast closer is too accommodating in KCl to respond to blunted shock (9). See text.

stant of accommodation, λ, was about 125 ms (picture 2). The fiber fired multiple discharges with slightly stronger stimulation, 20 mv, picture 3, even with the capacity in the circuit. After about 5 minutes in 2.5 × KCl in sea water the threshold had fallen to about 9 mv, a 3 mv decrease from the initial value as indicated by the dashed line, picture 4, and the spike amplitude was only 20 mv. When the 0.05 μf capacity was in the circuit, only one spike was elicitable even with very high voltage, picture 9, and threshold was raised

to 22 mv and was 10 ms. The lower row of pictures, 6–9, show the effect of the KCl sea water on the fast closer fiber. The initial rheobase value for this fiber was 18 mv, the large action potential spike measured 75 mv from tip to tip, picture 6, and with the 0.05 μf condenser in the circuit the threshold was 42 mv and λ then was 11.2 ms, picture 7. The spike appears much larger in this recording (7) because it is summating with the second response of the slow closer. The total voltage was 110 mv. The fast closer fired non-repetitively. After 5 minutes in 2.5 $\times$ KCl in sea water a 60 mv spike could be evoked only by a rheobase voltage of about 50 mv, picture 8, but could not be elicited by 400 mv when the condenser was in the circuit (9). The λ value was 2 ms or less at this time. The fibers were allowed to remain in this solution for 10 minutes and then normal sea water was admitted to the chamber and after two minutes, the fast closer rheobasic voltage was reduced to 16 mv and the λ value rose to 12 ms. The slow closer λ value recovered to 28 ms and the fiber fired repetitively once more.

The effect of only 1.8 $\times$ KCl in sea water on a slow closer fiber is illustrated in figure 4. There are 6 groups of three records each in this figure, each group having been recorded at the time in minutes after immersion in the solution represented by the figure above that group. Each group consists of a fast sweep speed (above) twin-beam recording which includes the action potential in the upper sweep and the 1 ms shock threshold superimposed on the rheobase (dashed line) on the lower sweep. Two similar recordings below in each group were made at slow sweep speed, one with blunted shock on the left and the other with no capacity but high resistance in the circuit on the right. The results are listed in table 1.

After two hours the action potential was reduced 70% of the original magnitude. These results were only partly reversible due to the exceptionally long exposure to high KCl concentration. Notice that at time 0 min. the rheobase was 50 while the threshold for 1 ms shock was 73 or 46% higher

than rheobase, but at time 120 min., the threshold for 1 ms was 200 mv only 18% above rheobase of 170.

Another series of records taken from a medial giant fiber, 180 μ diameter, which was exposed to 2 × KCl for 60 minutes are shown in figure 5. From top to bottom in each of the 4 groups of tracings is shown the action potential elicited by the blunted shock (dashed line) then the spike slightly to

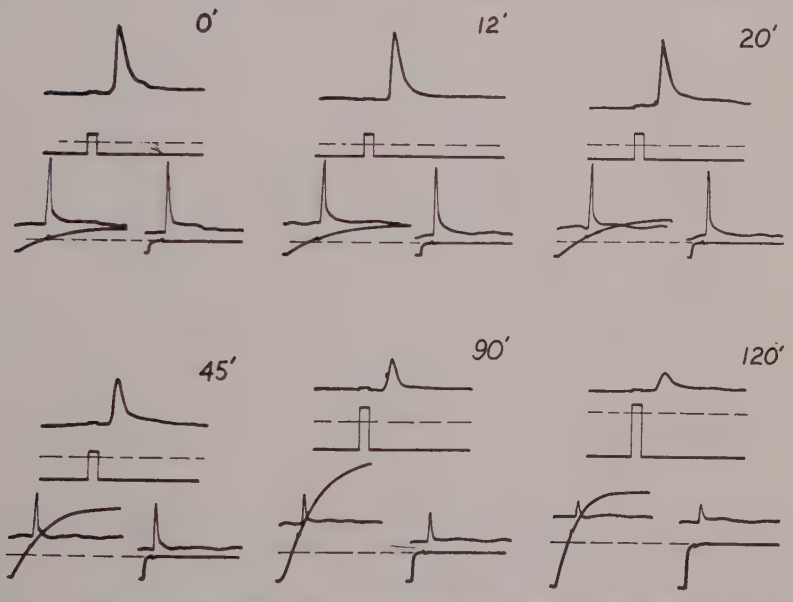

Fig. 4 Action potential and stimulus recordings from a slow closer in 2 × KCl in sea water. See text.

TABLE 1

TIME	RHEOBASE	THRESHOLD 1 MS SHOCK	% DIFFERENCE $(\frac{T-R}{R} \times 100)$	K	λ	(μF IN CIRCUIT)
min.	*mv*	*mv*	*%*		*ms*	
0	50	73	46	1.1	8	0.03
12	57	88	54	1.2	5	...
20	68	104	53	1.3	3.5	...
45	84	116	38	1.7	2.8	0.02
90	117	150	22	1.8	1.8	·0.01
120	170	200	18	1.9	1.7	

the left stimulated by threshold DC pulse both at slow sweep speed. Below these and recorded at fast sweep speed are first the spike responding to 1 ms shock and then slightly to the right due to the latency or utilization time, the spike evoked by rheobase stimulation and below are the respective stimulus pulses, 1 ms and rheobase. The values of the excitability constants are given in table 2.

There was little change in either the threshold or in the k value for 30 minutes but accommodation increased and the action potential amplitude decreased during this period. After

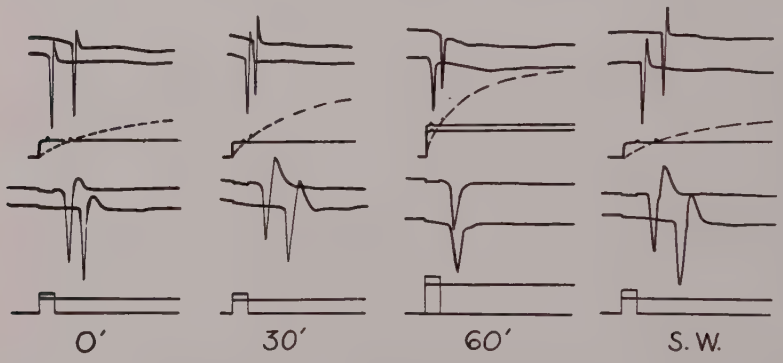

Fig. 5 Action potential and stimulus recordings from a medial giant 180 μ fiber in 2 × KCl in sea water. Four recordings were taken at each time period. At zero time, left group top to bottom, spike evoked by blunted pulse, spike evoked by rheobase square pulse both at slow sweep, spike elicited by threshold 1 ms shock and spike elicited by rheobasic DC. Other recordings taken after 30 and 60 minutes in the excess KCl and then in sea water (S.W.) showing recovery.

TABLE 2

TIME	RHEOBASE	THRESHOLD 1 MS SHOCK	% DIFFERENCE $(\frac{T-R}{R} \times 100)$	SPIKE SIZE	K	λ	(μF IN CIRCUIT)
min.	*mv*	*ms*	%	*mv*		*ms*	
			1.8 KCl				
0	75	92	23	45	1.6	6.0	0.03
30	73	94	29	37	1.5	3.5	
60	150	180	20	30	1.8	2.3	0.02
			Sea water				
5	80	115	43	45	1.2	6.8	0.03

one hour the spike amplitude was reduced by one third, broadened about 100% normal size, and monophasic. The rheobase latency was reduced almost 50% and the threshold for both rheobasic and 1 ms shock was about doubled. The k value was larger than originally and the accommodation time constant was reduced 60%. As in the opener experiment (fig. 3b) blocking occurred between the recording interface and the recording electrode so that the action potential

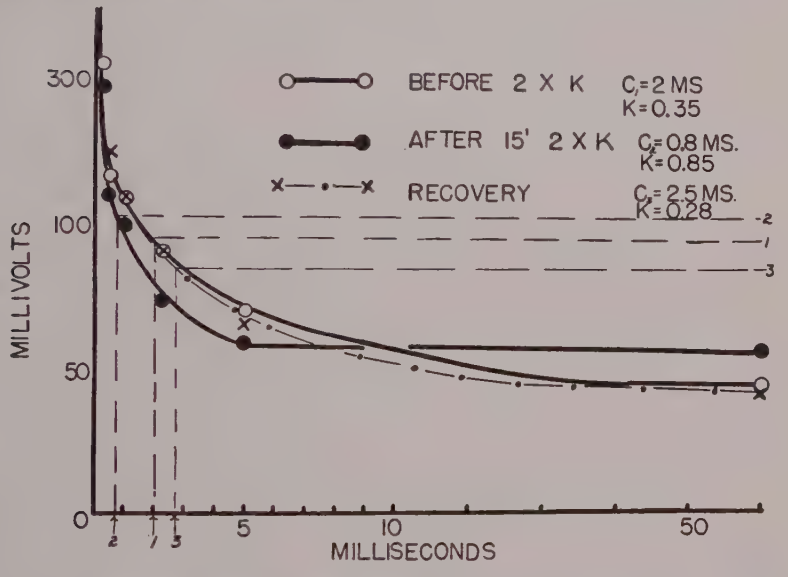

Fig. 6 Strength-duration curves obtained from an opener fiber in sea water (open circles) after 15 minutes in 2 × KCl in sea water (solid dots) and in sea water again (X's).

became monophasic. Reversal of all changes was complete after 5 minutes in sea water. The data show that excess KCl in sea water increases both the threshold, and k value, while decreasing the λ value. It is interesting to note that in this case a very large, non-repetitive type fiber, the 1 ms shock threshold at time 0 is only 23% above rheobase and at 60 minutes is 20% above rheobase. The per cent difference between the 1 ms shock threshold and rheobase is constant at all times and the same as the difference between the 1 ms

shock threshold and rheobase (18%) in the repetitive type fiber *after* the effect of KCl has taken place and the fiber is no longer repetitive.

The decrease in difference between short shock threshold and rheobase was due to another effect of KCl, i.e., raising the threshold and enlarging k, thus changing the shape of the strength duration curve. Typical strength duration curves taken on an excellent opener fiber preparation in sea water, after 15 minutes immersion in $2 \times$ KCl concentration and again in sea water, are illustrated in figure 6. The chronaxie and k values calculated from these curves are listed in the figure.

The mean threshold change with standard error in per cent plotted against time for all fibers tested in all concentrations of excess KCl is shown in figure 7, upper graph. There is an initial 5 to 10% decrease of the threshold value, the so-called hyperexcitable state (Lehman, '37), and this is followed by a steady rise. The standard error at the 4-minute mark is large because the thresholds of many of the fibers subjected to the $5 \times$ KCl concentrations in sea water were extremely high at this time, and conduction in a number of fibers was already blocked. At the same time the thresholds for some fibers subjected to only $2 \times$ KCl were still lower than normal and so there is a large scattering of threshold values at this time.

The effect of the KCl solution was so rapid that it was hard to tell in many cases whether the fiber passed through a "hyperexcitable" state or not in the first few minutes. When the fiber was returned to sea water (vertical dashed line), the initial mean threshold values were 30 to 40% below the original normal value and rapidly rose to the normal value. The potassium gradient across a fiber membrane was a bit low initially and the fiber slightly depolarized after flushing the chamber with sea water especially in those experiments with the $5 \times$ KCl solutions.

The lower graph shows the per cent threshold change of three fibers, fast closer (solid dots), slow closer (open circles),

and the medial giant fibers (shaded circles), when these fibers were exposed to 2 × KCl in sea water. The small arrows indicate where the KCl solutions were replaced by fresh 2 × KCl solutions. Both the fast closer and medial giant fiber thresholds initially decreased, then rose, while the slow closer threshold in this case did just the opposite. The early threshold changes with low amounts of KCl added to sea water were quite variable and somewhat unpredictable.

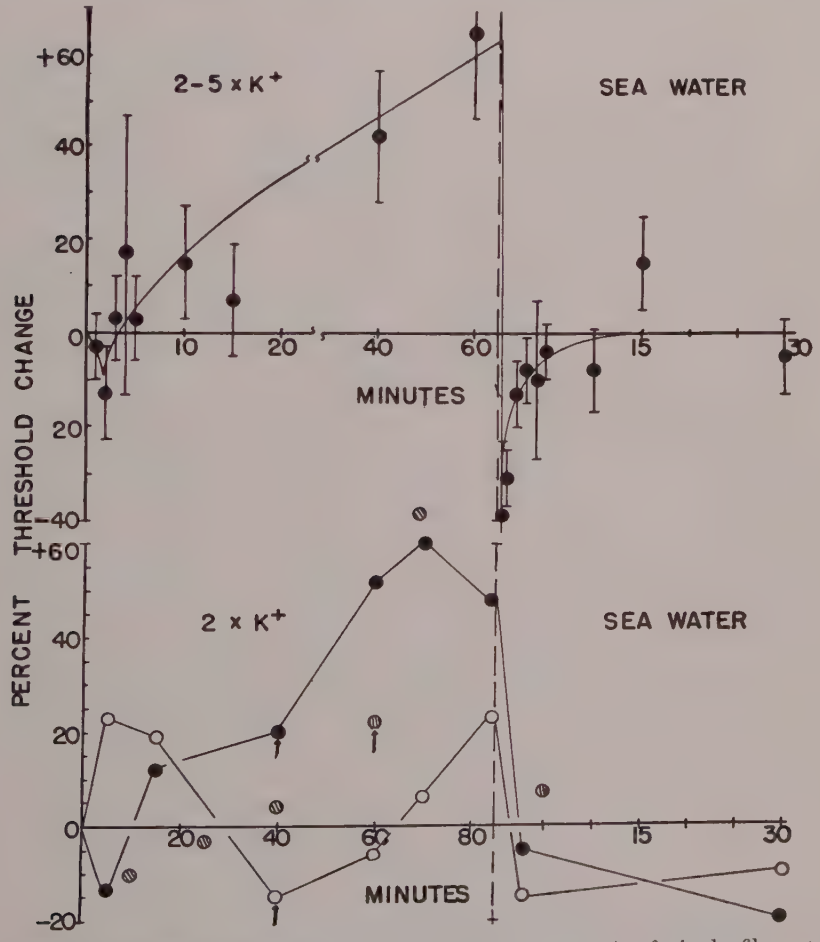

Fig. 7 Per cent of change (±) of threshold (ordinate) of single fibers exposed to 2–5 × normal KCl in sea water in minutes (abscissa). See text.

The mean change and standard error of k values in per cent for all nerves and in all concentrations of KCl in sea water is plotted against time in figure 8, upper graph (solid dots). The small circles show the per cent changes of k value in fibers in 5 × KCl and the X's show the per cent changes in fibers in 2 × normal KCl. The per cent increase

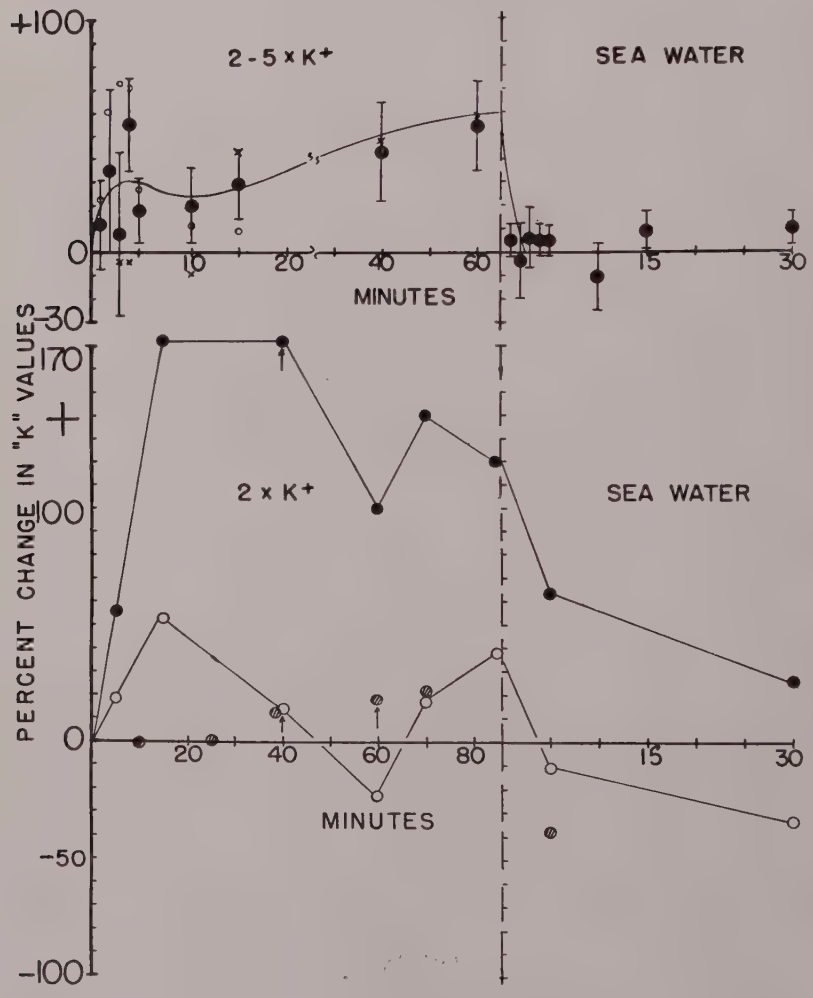

Fig. 8 Per cent change (ordinate) (±) of "k" values of single fibers exposed to 2–5 × normal KCl in sea water in minutes (abscissa). See text.

in k values in the first 5 minutes was large (+ 70%) with 5 × KCl, but was almost negligible with only 2 × KCl. Peculiarly, k values in 5 × KCl decreased again to almost normal value in 15 minutes, while at this time the k values of fibers in 2 × KCl had increased and continued to do so. The reversal of the initial k value change may be simply a result of a large scattering of k values. However, a very similar variation in the k values of frog sartorius muscle subjected to excess KCl has been reported (Blair et al., '38). It means the strength-duration curve is shifting to the right relative to the axes. It is clear from the graph that the k value does increase as much as 50% in a fiber treated with excess KCl and that this change is rapidly reversed in normal sea water.

A statistical test of the differences of the k values of fibers in sea water and in high KCl concentrations resulted in a P value of less than 0.01 which means that k values from fibers treated with KCl are significantly greater than k values from fibers in sea water.

In the lower graph the per cent change of k values of the three fibers, fast closer (black dots), slow closer (open circles), and medial giant (shaded circles), are plotted against time after the fibers were exposed to 2 × normal KCl and sea water. There is a marked increase in the k value of the fast closer, + 170% as compared to 50% increase in the slow closer fiber k value in the same period of time, i.e., 15 minutes. Subsequent changes of solutions, small arrows, had little effect, but normal sea water reversed the effect of KCl completely and rapidly. A statistical test of the differences between changes of k values of fast closer fibers and slow closer fibers in all concentrations showed that the k values of the fast closers were increased significantly more than those of the slow closers. Not only is the k value of the nerve fiber increased by excess KCl, but the effect is significantly more pronounced in the larger non-repetitive firing type fiber than in the smaller repetitive firing type.

The mean per cent change with standard error in the accommodation time constant, λ, for all fibers subjected to all

concentrations of excess KCl in sea water is plotted against time in figure 9, upper graph, large solid dots. There was a most remarkably sudden 90% to 100% decrease in λ within three minutes. The per cent changes due to data from fibers in 5 × KCl are indicated by the small circles. The λ values of fibers in 2 × KCl were decreased 50–60% in 5 minutes

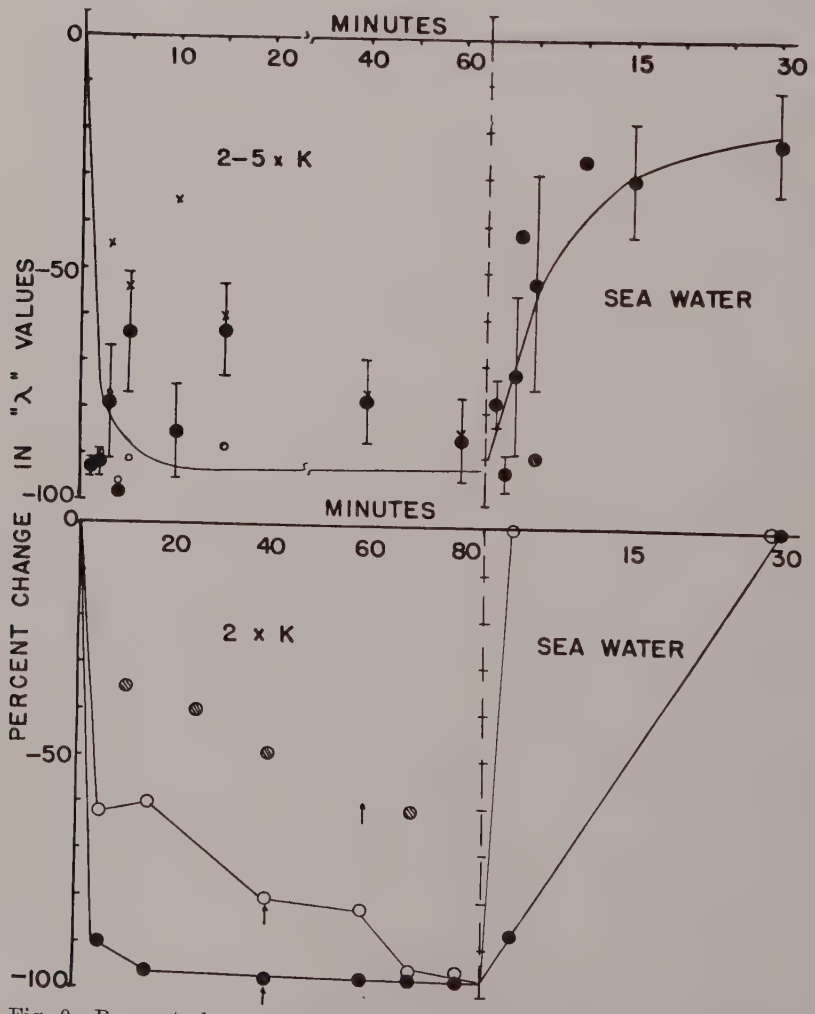

Fig. 9 Per cent change (ordinate) (±) of λ values of single fibers exposed to 2–5 × normal KCl in sea water in minutes (abscissa). See text.

and 90% or more after one hour (X's). The change was reversible in sea water but it is interesting to note that recovery of normal λ values was slower than the reversal of k values or threshold values. A normally repetitive firing fiber which in high KCl became unable to fire repetitively, upon return to normal sea water regained the original normal and even 30–40% below normal rheobase value but remained incapable of firing more than one impulse per stimulus for some time because of the lag in the recovery time of λ to a high enough value to allow repetitive firing to take place.

The effect of $2 \times$ KCl in sea water in per cent change of the λ value of the fast closer (solid dots), slow closer (open circles), and medial giant fibers (shaded circles) is plotted against time in the lower graph of figure 9. The effect was most pronounced on the fast closer which happened to be an unusually non-accommodating fast closer fiber with an initial λ value of about 100 ms. This value was reduced to 10 ms or 90% in 5 minutes and 97% in 15 minutes. After 80 minutes in high KCl sea water, the fiber was returned to normal sea water. In less than 5 minutes the k value had reversed 50%, figure 8, and the rheobase had reversed 105% or was actually 5% below normal, figure 7, but the λ value was still 87% below normal. The fiber responded non-repetitively although rheobase was below normal. Repetitive discharges appeared much later when the threshold was considerably higher, near normal, and λ had increased. The λ values of the slow closer, figure 9, were also very greatly decreased but not so rapidly in this case as in the case of the fast closer fiber. The reversal of this change in λ was very rapid and complete in both the slow closer and medial giant fibers in this experiment. A tremendous decrease in the time constant of accommodation, λ, is the effect of excess KCl. Non-accommodating nerve fibers become rapidly accommodating and accommodating nerve fibers become so extremely rapidly accommodating as to become difficult to stimulate at all.

The changes in the excitability constant k and λ were observed simultaneously by making use of the dual shock method

(Erlanger and Blair, '31; Parrack, '40; Monnier, '52; and Le Fevre, '50). This technique was applied in three experiments to opener fibers. The results of one such test are shown in figure 10. The data shown by the upper curves were taken

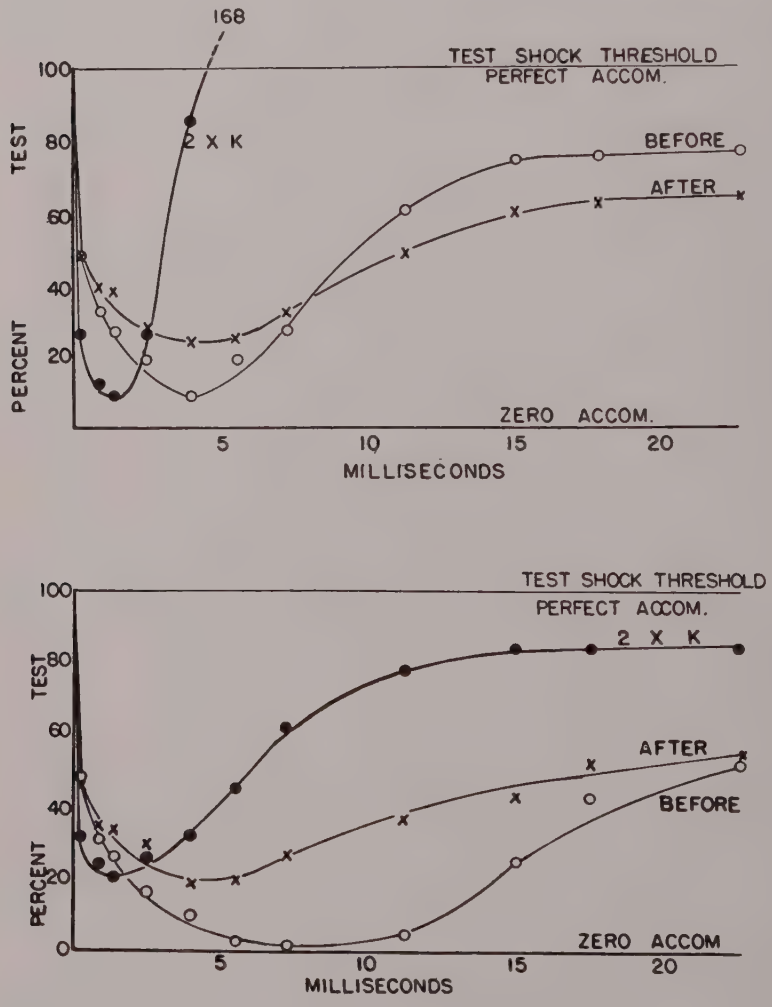

Fig. 10 The effect of 2 × KCl in sea water on an opener fiber excitability tested by the dual shock method. The upper graph shows the curves obtained in sea water (open circles), in 2 × KCl in sea water (solid dots) and in sea water again (X's). The fiber was not firing repetitively at the start of this run but began during recovery. After 30 minutes in sea water the fiber was highly repetitive and the test repeated, lower graph. See text.

first, and 30 minutes later the data for the lower curves were obtained from the same fiber. The method requires the superposition of a short 0.2 ms test shock for stimulation threshold determination upon a subthreshold, approximately 90% threshold, long duration DC voltage at various times after the start of the subthreshold voltage. The subthreshold voltage causes a buildup of excitation which will occur with the rate k and this rise in excitation can be recorded as a decrease in the 0.2 ms shock threshold shortly after the onset of the conditioning subthreshold voltage. Thus the test shock threshold value in per cent of normal (ordinate) is plotted against time in ms of application after the start of the subthreshold voltage.

In the absence of accommodation — zero accommodation — if the conditioning voltage is X% rheobase, excitation will be raised to a limiting value which will require an additional test shock voltage equal to (100 — X)% test shock threshold to excite. Since these limits are different with different conditions, this limiting value, 100% minus X%, has been adjusted for every curve to zero so that the curves are all plotted to the same coordinates. In most cases the DC subthreshold voltage was about 80% threshold so that the limit of zero accommodation was about 20% test shock threshold value when measured without the DC present. The upper limit or perfect accommodation limit will vary depending on the lower limit, because a perfectly accommodating fiber has been defined (Hill, '36) as one which reacts to an applied DC subthreshold voltage by raising the threshold so that the test shock required to excite is always equal to its value obtained in the absence of any DC voltage or a full 100% value. This limit has been adjusted for all curves to the 100% mark on the ordinate. Sometimes accommodation is so great that a new limit above perfect accommodation is approached. This is called negative accommodation and signifies a pronounced depression of the state of excitation of the nerve fiber.

The subthreshold voltage will cause the excitation to rise or threshold to fall at a rate k, or with time constant $= \frac{1}{k}$ ms. The value of 1/k of each test run can be roughly approximated from the curves by noting the time required for the threshold to fall to $1/e^{th}$ or about 31% original value. The time required for the threshold to rise to 100% less $1/e^{th}$ or 69% original value gives a rough approximation for λ. At least any changes in the two time constants can be compared simultaneously in this manner.

The opener fiber preparation became non-repetitive during the trial runs before any data were taken by the two-shock method. The first data obtained from this fiber in the non-repetitive state is plotted in the upper graph in figure 10 (open circles). The 1/k value was about 1 ms, so $k = 1$ and the λ value was about 15 ms which accounts for the non-repetitive response of this fiber (Wright and Adelman, '54). When exposed for two minutes to $2 \times$ KCl the curve obtained (solid dots) was much different than before. The threshold fall was more rapid and the threshold rise was extremely fast. The 1/k value was about one-fourth the original value so k was about 4 and λ was reduced to less than 5 ms. It will be noted that this curve overshoots the perfect accommodation limit. It approaches another one at 168% threshold value as indicated by the number on the curve. Under the influence of $2 \times$ KCl the fiber had developed negative accommodation and was in a very depressed state of excitation. The fiber recovered after three minutes in sea water (curve with X's) to a higher excitatory state than the original one. 1/k was about 2.5 ms or k was 0.4 and λ was greater than 25 ms. Now the fiber began responding to DC stimulation with multiple discharges as it should normally when stimulated with direct current. After a 30-minute rest interval in sea water the tests were repeated and the results are shown in the lower graph. At the start (open circles) 1/k was about 1.7 ms or k was about 0.6 and λ was much greater than 25 ms. In fact there was zero accommodation for a period of 10 ms. After 5 minutes in $2 \times$ KCl (solid dots)

1/k was about one-fourth, the k value was approximately 4 and λ was reduced to 8 ms. The fiber was once again unable to respond repetitively. This time, however, there was no negative accommodation. After 5 minutes in sea water (curve with X's) 1/k became equal to about 2 ms or k was reduced

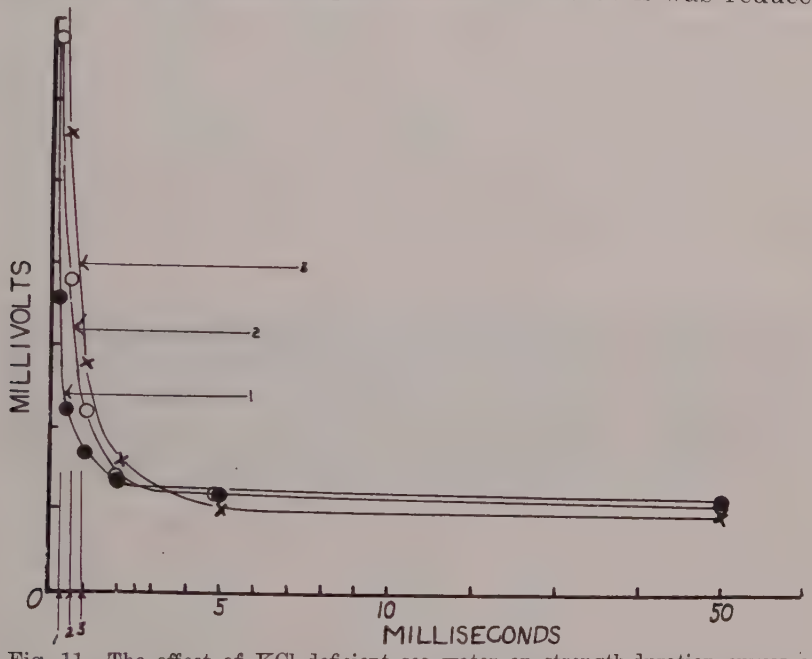

Fig. 11 The effect of KCl deficient sea water on strength-duration curves of a fast closer fiber. The rheobase values which increased continually have been adjusted to about the same level so that the curvatures of the three curves may be compared. At time zero (solid dots), rheobase flat and curve (1) is acute, bends up at 2 ms. After 5 minutes (open circles) rheobase flat but curve bends upward at 2.5 ms. After 10 minutes the curve bends upward at 5 ms. The fiber blocked irreversibly right after this curve data was taken. The figures indicate the approximate chronaxie values. See text.

to 0.5 and λ increased to 25 ms. The fiber fired repetitively when excited. The three experiments produced the same results. Two times KCl in sea water definitely caused an increase in k value and decrease in λ value. Also, there appears to be very little if any accommodation in good opener fibers under normal conditions and it is apparent from the results in figure 11 that a fiber in the non-repetitive firing state is

considerably more affected by KCl than a fiber in the repetitive response condition. This we have already noted as a difference between the effect of KCl on the large, non-repetitive fast closer fibers and the smaller repetitive slow closer and opener fibers.

Six experiments carried out in which single fibers were exposed to KCl-free artificial sea water produced results similar to those for excess potassium, except that sooner or later an irreversible conduction block was produced. This happened within 5 minutes after immersion in the test solution in two opener fiber preparations. The action potential began decreasing in size immediately and in two minutes

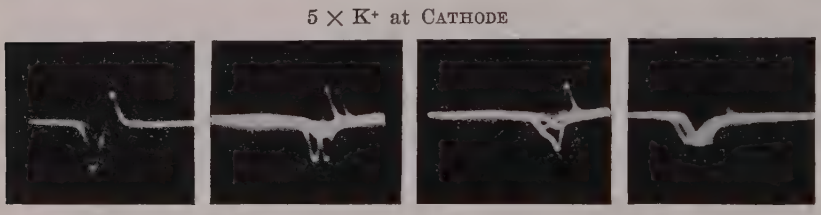

Fig. 12 Local and spurious responses obtained from the cathodal region of a single fiber in 5 × KCl in sea water and identical to those obtained from the cathodal region of a refractory or fatigued fiber.

was reduced to a local response which soon disappeared. In one case the k value was obtained and it had decreased about 50% before block. Both fibers became non-repetitive indicating an accommodation increase. In the other 4 experiments λ was always reduced in value while k decreased in two and increased in the other two. The most startling result was that all experiments were terminated by the onset of irreversible conduction block.

Three strength duration curves taken at different times in KCl-free sea water have been adjusted to about the same rheobase value so as to be superimposed (fig. 11). During the early period after immersion of the fiber in KCl-free sea water the curves became slightly less acute with more curved extensions similar to those curves now associated with the

small repetitive type nerve fiber. From these few experiments with KCl-free solution, no definite conclusions can be drawn, except that apparently in the absence of potassium the excitation and conduction mechanism becomes irreversibly inactivated.

The effect of 5 × KCl on a fiber raised almost entirely in oil so as to record from the cathodal area is shown in figure 12. The spike is superimposed on the local response. The latency of the spike increased significantly and the amplitude of the local response was augmented as the KCl effect progressed (Marmont, '42). Later the local response also died out. These effects were reversible. These recordings are very similar to recordings from the cathodal region of a refractory or fatigued fiber (Wright and Coleman, '54).

DISCUSSION

It has been possible by merely adding KCl in excess of normal concentrations to the surrounding medium, sea water, to transform the response pattern of highly repetitive firing non-accommodating lobster nerve fibers to that of accommodative non-repetitive firing ones. After a short time in high KCl the behavior of opener fibers is the same as the behavior of fast closer fibers or giant fibers of the ventral nerve cord. The small fiber strength-duration curve shape with low rheobase and long chronaxie or small k values becomes identical in curvature to the strength duration plot of the large fibers or fast closers with higher rheobase and short chronaxie or large k values. But this change does not occur immediately. Often during the early stages of exposure to high KCl the fiber rheobase is lowered while the k value increases and although the curve becomes more acute with flatter extensions, it is lowered relative to the voltage time axes. The curve also may assume this position relative to the axes during the initial recovery period especially after very high concentrations of KCl have been used. Although this transient lowering of the rheobase has been defined as a hyperexcitable state during which spontaneous discharging

may occur (Lehman, '37), there has been no indication of such autorhythmic firing by fibers in this state in the present work. Neither accommodating nor non-accommodating fibers show an increase in λ at any time in excess KCl. In fact, as has been shown, quite the contrary is always the case. Furthermore, excess KCl is antagonistic to spontaneous repetitive discharging whether it be evoked by calcium deficiency in frog nerve, acetylcholine in mammalian post ganglionic fibers (Brink, Bronk and Larrabee, '46) or natural causes in the crustacean ventral nerve cord (Prosser, '42). And the reason is that the one really consistent and immediate effect of KCl excess is inducement and enhancement of accommodation. This result bears out the observations of Solandt ('36) and Parrack ('40) on frog nerve. Repetitive firing does not occur spontaneously nor can it be induced electrically in a fiber with an accommodation time constant value less than about 22 ms (Wright and Adelman, '54), and λ values of fibers in excess KCl are in general 10 ms or much less. Consequently, the early effect of KCl may reduce the rheobase slightly but it does not produce a hyperexcitable state in the true sense of the word. The similarity of recordings from KCl treated fibers and recordings from fibers either fatigued or refractory (fig. 12 above, and Wright and Coleman, '54) is indicative that the refractory state, as has been suggested by Shanes ('49), and the fatigued state of excitability and action potential are due to the outflux of potassium ions during the response and the collection of these ions in the immediate surround of the fiber. Not only is repetitive firing absent, but local responses become evident (Marmont, '42; Wright and Coleman, '54) and spurious spikes appear (Hodgkin, '38; Wright and Coleman, '54). Fatiguing or deteriorating fibers develop substantial accommodation (Wright and Adelman, '54). When the fiber has been in excess KCl solution for longer times, the rheobase is raised and the k value may increase more or even decrease as reported for frog sartorius muscle by Blair ('38). The

strength-duration curve is then moved upward and to the right relative to the axes.

The threshold of a non-accommodating fiber is stationary and may be reached by a very slow buildup of excitation after a long latent period, but the threshold of an accommodating fiber moves rapidly away from an excitation process rise and so the rise must take place with speed if it is to reach the elusive threshold and excite the fiber at all. Consequently the latency of a highly repetitive firing fiber, Group I, is much longer than the latency of a non-repetitive firing fiber, Group III, as also reported by Hodgkin ('48), with rheobasic stimulation. The effect of excess KCl on the excitability of a non-accommodating fiber is illustrated diagrammatically in figure 13. In the upper two drawings, A, λ is infinite, the threshold is constant, top drawing, and is reached at (1) after a long latency (L) by the excitation buildup induced by rheobasic voltage (1) lower drawing. A slightly shorter shock (2) lower picture, must be stronger to raise the excitation process to threshold at (2) and so on for shocks of still shorter duration 3, 4, and 5. A smooth curve can be drawn through the points 1–5 and its curvature is the same as the strength-duration curve of a small non-accommodating fiber (Easton, '52; Wright and Coleman, '54). If the shocks stronger than rheobase, 2–5, are increased in duration, the excitation process will overshoot the threshold value (dashed lines) and repetitive firing will ensue during the overshoot period until the stimulation is discontinued. After treatment with KCl the λ value is reduced to say 10 ms or about 5 k, lower drawings (B). The threshold of this fiber tends to "escape" as the excitation process is raised and rheobase shock strength (1) will never excite. A stronger stimulus (2) will excite and it does not matter if this voltage is maintained for very long times or not for it will only just "catch" the threshold once at point (1) after a very short latent period (L), and never at any later time. There will be no repetitive firing at rheobase and even with stronger shocks (2, 3) the overshoot period

is so short as to allow for very little or no repetitive firing either. The shape of the curve through points 1–3 is the same as that of an accommodating fiber such as a fast closer or a potassium treated opener. This explains why the latency of an opener fiber is so markedly reduced by potassium as shown in figure 3a and why the strength duration curve shape changes in solutions with large amounts of the ion.

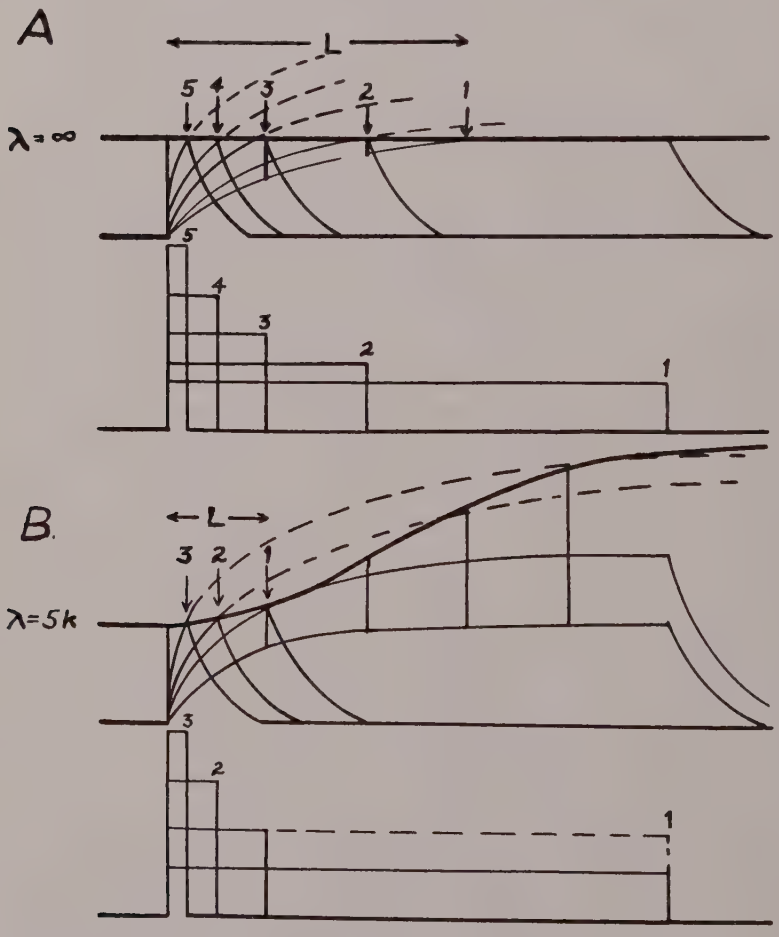

Fig. 13 Diagrammatical explanation of changes produced by excess KCl on nerve fiber excitability including latency, strength-duration curve shape and repetitive response.

A decrease in velocity of conduction in potassium treated fibers was not apparent if measured by the latency from stimulus to the foot of the spike until just before block (figs. 3a, 12), but if the peak of the spike was used as an end point of the latency (Taylor, '53) then a velocity reduction was noticeable. The rate of rise of spike potential is decreased considerably (figs. 3a, 4, 5) by potassium as reported previously for frog nerve as decreased anstiegeschwindigkeit by Rosenberg ('30), and as is evident in squid giant axon action potentials in $2 \times$ KCl (Hodgkin and Katz, '48, fig. 12). An anodal current should increase the rate of spike-rise but lengthen if anything the real latency measured at baseline, and therefore should increase the reduced conduction rate by the one factor alone. As Taylor points out the limit of the effectiveness of an anodal current in restoring the reduced velocity to normal will be the velocity value, somewhat reduced, from normal, obtained in nerve subjected to anodal polarization alone.

The amount of potassium emitted per impulse by a 40 μ axon is about 2×10^{-14} moles/cm (Hodgkin and Huxley, '46). The thin film of saline, 3 μ thick, surrounding such an axon raised in oil contains about 3×10^{-11} moles potassium per centimeter. Since reabsorption is slow, approximately 1500 impulses will emit a like amount into the surround and double the concentration. Thus an opener fiber which is 40 μ in diameter and which fires repetitively at a frequency of 50–100 spikes per second should accomplish by its own discharges a doubling of potassium concentration outside the membrane and thereby produce substantial accommodation in 15 to 30 seconds. The accommodation so produced should be ample to shut off the train of spikes. It is extremely interesting that when the claw or limb is cut from the animal this mechanical stimulation elicits an opening reaction of about 15 to 30 seconds' duration. The smaller slow closer fiber, discharges for a shorter period of time *in vivo* possibly because the frequency of discharge is higher. It runs beside, in fact adheres to, the fast closer so it may be considerably

influenced by potassium leakage from this large fiber. We may assume that due to the larger size and surface area, considerably more potassium is lost by fast closer fibers and other larger giant fibers per impulse, but a direct relationship of potassium outflux to size will not account for the marked difference in accommodation between large and small fibers. A fast closer, only twice the diameter of the opener, in the claw should fire repetitively for 5 to 10 seconds, but it responds only once or a few times in less than a second. The λ value of the medial giant fiber is about 5 ms normally and of the squid giant axon less than 2 ms. Consequently, it must be assumed that these fibers leak potassium at a relatively high rate in the passive state *in vivo*. The crustacean nerve fiber *in vivo* is buried in connective tissue with poor circulation around and so it may be considered to exist with only a thin film of body fluid around it and hence should act much as it does when isolated in oil. The truly large giant fibers must exist in a depressed state of excitability bordering on block probably due to a high rate of potassium leakage. This would account for the more pronounced effect potassium has on the large fibers and also the more rapid fatigue and deterioration occurring in these fibers *in vitro*.

The actual mechanism by which excess KCl causes the loss of excitability is not known, but the recent work of Hodgkin and Huxley ('52a, b, c, d) sheds some new light on the problem. Just as reported here, these authors observed the extinction of the action potential or its marked reduction by small excess concentrations of KCl even though the membrane potential was decreased only slightly. This was attributed to the inactivation of the sodium carrier system and it has been shown by the voltage clamp method that an increase in the potassium conductance is responsible for this inactivation. The potassium conductance is increased by a sustained cathodal current which accounts for the inactivation (Schoepfle and Erlanger, '49) or accommodation occurring with the long duration subthreshold stimulation. And it has been shown that conductance across a giant axon

membrane is increased by excess KCl (Hodgkin, '46; Shanes, '53).

Perhaps speculation is out of place at this early stage of the work, but it certainly is an interesting possibility that adaption of sensory endings and fibers in all forms is automatically induced by the long trains of impulses from the fibers and endings themselves. Indeed the larger fast pain fibers, delta fibers, respond only briefly while the slow adapting smaller C fibers respond for long times. This difference seems very similar to the one found between fast and slow closers or openers in the crustacean limb. But it should be emphasized here that the potassium effect is only a part of the phenomenon, since fibers deprived of calcium can fire indefinitely. Evidently the amount of potassium accumulated by impulse leakage alone is not enough in these cases to suppress this reaction.

SUMMARY

The effect on 47 lobster single motor axons of different concentrations above and below normal of KCl in sea water has been investigated.

Excess KCl caused:

(a) Increase of as much as 70% in the k value which sometimes was followed by a decrease to normal with high concentrations of KCl;

(b) A rise in threshold, sometimes preceded by a transient subnormal phase;

(c) An immediate 90–100% reduction in the time constant of accommodation, λ. Repetitive firing was abolished;

(d) A marked decrease in the action potential amplitude;

(e) A slight decrease in conduction velocity;

(f) A pronounced reduction of latency with rheobase stimulation.

All these effects were rapidly reversible in normal sea water. KCl lack caused an irreversible conduction block in 6 experiments.

It is concluded that excess KCl in sea water transforms the response pattern of a repetitive type fiber (opener or slow closer) to the response pattern of a non-repetitive type fiber (fast closer or medial giant). In excess KCl the strength-duration curves of small repetitive fibers become shaped like curves obtained from normal large non-repetitive fibers, and the λ values of all fibers become very small.

The pronounced enhancement of accommodation by potassium prohibited the appearance of a so-called hyperexcitable state in the sense that spontaneous or repetitive discharges were never evokable from a potassium treated fiber.

It is suggested that the very large crustacean fibers due to excessive potassium leakage exist normally in a state of relatively low excitability compared to the small fibers.

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THE ENERGETICS OF CELL DIVISION: EFFECTS OF ADENOSINE TRIPHOSPHATE AND RELATED SUBSTANCES ON THE FURROWING CAPACITY OF MARINE EGGS (ARBACIA AND CHAETOPTERUS)[1]

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FOUR FIGURES

The gel contraction hypothesis, as formulated in relation to cytokinesis by Marsland ('50) and by Marsland and Landau ('54) forms a basis for the present experiments. According to this view, the furrowing potency in animal cells depends upon the structural state (and hence the contractile capacity) of the cortical layer of gelated cytoplasm, in the region bordering the cleavage furrow. In testing this hypothesis, it is therefore important to ascertain whether or not all conditions tending to change the structural strength of this gel also produce a corresponding change in the furrowing performance of the cell.

It now seems well established that protoplasmic gelations generally display the Freundlich ('37) criteria for a type II system (see Marsland and Brown, '42 and Marsland, '50). Such gelations, being endothermic, tend to be strengthened by increasing temperature (within physiological limits); and since a volume increase ($+\Delta V$) occurs when this type of system undergoes gelation, such gels are weakened when

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subjected to increasing pressure. Accordingly, it has been found that the furrowing potency of various eggs increases at higher temperature and decreases at higher pressure, in correspondence with changes in the gelational state of the cytoplasm bordering the furrow which have been demonstrated by the centrifugal method under corresponding conditions (Marsland and Landau, '54).

Granting that the formation of a protoplasmic gel network and its subsequent contraction represents a mechanism for converting metabolic energy into mechanical work, it becomes important to ascertain what specific metabolic substances and reactions are utilized by the cell in providing energy for the endothermic phases of the gelation process. The reports of Marsland and Brown ('42), Kriszat ('49), Runnstrom and Kriszat ('50), Loewy ('52), Hoffman-Berling and Weber ('53) and Hoffman-Berling ('54), all tend to suggest that the high energy components of the adenosine triphosphate system are involved. Consequently, it was decided to investigate how the furrowing potency of marine eggs might be affected by providing additional ATP through the medium of the surrounding sea water.

METHODS

Chemicals. The adenosine triphosphate, obtained from The Schwarz Laboratory, Mount Vernon, N. Y., was of two grades, namely chemically pure and chromatographically pure; but no differences in the physiological activities were discernible. It was found, however, that any given sample, even though kept in a dessicator in the freezing compartment of a refrigerator, tended to lose some activity in the course of 4–5 weeks. Consequently, new samples were obtained every three weeks and fresh stock solutions, made up directly in sea water, were prepared every day and kept in the refrigerator, pending use in each experiment. The concentration of ATP in the final immersion media of the various experiments ranged from 0.0005 M to 0.001 M, and these solutions showed

no measurable departure from the pH value of normal sea water.

Adenosine-5-mono-phosphate (AMP) was purchased from Bischoff Laboratories, Ivoryton, Conn. under the commercial name MY-B-DEN, in the form of ampules, each containing 20 mg AMP in 1 cm^3 of distilled water. The final dilutions with sea water were of such magnitude that the osmotic effects were negligible. Fresh sea water-AMP solutions were made up daily and kept in the refrigerator pending use.

The microscope-pressure chamber. This chamber (see Marsland, '50) permits the cells to be observed at magnifications up to 600 diameters while they are subjected to pressures up to 16,000 lbs./$in.^2$. Such continuous observation is essential since, typically, the effects of pressure are rapidly reversible following decompression. The pressure in this system was built up by means of successive strokes of a pump handle, at the rate of 2000 lbs./$in.^2$/sec.; and decompression, either partial or complete, was achieved almost instantaneously, by means of a release valve.

The temperature control chamber. This chamber, which was described by Marsland ('50), provides a housing for the microscope, the microscope-pressure chamber and the pressure-centrifuge equipment (see below). It is equipped with cooling and heating units, a circulating fan and a bimetallic thermoregulator, which permit the temperature to be set at any point between —5° and 60°C., with a maximum variation of the internal air temperature of $\pm$ 0.3°C. This equipment makes it possible to observe the eggs directly while they are subjected to various temperatures and pressures in the specified ranges and to obtain centrifugal measurements of changes in the structural state of the cortical protoplasm throughout the same ranges of temperature and pressure.

The pressure-centrifuge. Measurements of the consistency (gel strength) of the gelated cortical protoplasm, as a function of both pressure and temperature were made with a pressure-centrifuge head similar to one described by Brown

('34). A needle valve makes it possible to seal one of the two chambers of this head after a certain pressure (up to 16,000 lbs./in.^{2}) has been built up; and then the whole head may be disconnected from the pressure pump and screwed to the shaft of the centrifuge motor. The other chamber of the centrifuge head contains the control cells at atmospheric pressure — so that the control and pressure-treated cells are simultaneously subjected to the same centrifugal force. These operations were performed very quickly. In fact, the centrifuging was started within a minute after the pressure was built up.

The centrifugal method of measuring the relative consistency (gel strength) of the gelated cortical protoplasm at various temperatures and pressures is based on the usual assumption that Stoke's law provides a useful approximation when applied to protoplasmic systems. Quite obviously the resistance to the displacement of visible granules through a gel cannot be regarded as an index of viscosity in a strictly physical sense. However, assuming that a uniform centrifugal force is used and that the density differential between the granules and the surrounding medium is not altered by the experimental treatment, it seems valid to take the centrifuge time required to produce a unit displacement of the granules through the gelated cortex of the egg as an index of the relative "consistency," "stiffness," or "strength" of this part of the protoplasm.

Measurements of the relative gel strength have now been made on numbers of different cells, including two species of Amoeba (Brown and Marsland, '36), two species of Arbacia (Brown, '34; and Marsland, '38 and '39a), and Elodea canadensis (Marsland, '39b); and the consistent pattern of all these data tends to justify the basic assumption involved.

Temperature control. Proper temperature equilibration was assured by keeping the pressure chamber and all glassware and solutions in the temperature control chamber for an adequate period prior to each experiment.

Maintaining the eggs at a fixed temperature while they were being put into the pressure-centrifuge chamber, compressed and centrifuged, required very careful attention. The pressure centrifuge head was kept in a water bath while the eggs were being introduced into the pressure and control compartments; and the pressure line was lead directly into the temperature chamber, so that no drift occurred while the pressure was being built-up, and while the head was being screwed to the motor shaft. Moreover, the refrigerating unit was turned on during centrifugation so that the heat generated by the rotation of the motor and head did not produce any significant elevation of the temperature. In fact, the maximum variation of air temperature in the thermal chamber during the centrifugation experiments was not greater than $\pm$ 1.0°C.

Control of centrifugal force. Subjecting the eggs to identical centrifugal forces at widely different temperatures also required very careful attention, since preliminary trials showed that the speed of the centrifuge motor fell off significantly at lower temperatures unless compensatory increases were made in the voltage of the power supply. In all the experiments, therefore, the centrifuge speed was determined stroboscopically (by means of a "Strobotac") and voltage adjustments to give the same speed at each different temperature were made with a variable transformer (Variac), powered from a 115 volt line. A voltmeter, placed between the transformer and the motor, permitted a continuous reading of the voltage. In all of the experiments, fluctuations in the power line during centrifugation were eliminated by means of a voltage stabilizer, placed between the power source and the variable transformer.

Other procedures. A shedding of the eggs of *Arbacia punctulata* was effected by an intracoelomic injection of 1 cm^3 of 0.52 M KCl. The shed eggs were then immediately washed twice by decantation and set aside as a stock suspension. Sperm, on the other hand, were obtained by removing the testes from the opened animal, after which the gonads were

rinsed quickly in sea water, and placed in a dry Syracuse dish as a stock of "dry sperm".

The techniques used for *Chaetopterus pergamentaceus* closely followed those described by Heilbrunn and Wilson ('48). In the case of Chaetopterus, however, eggs from a "stock suspension" could not be used in successive experiments, since they showed signs of deterioration within 30–40 minutes after liberation into sea water.

THE EXPERIMENTS

Concentration-time factors. Preliminary experiments, in which the concentration of ATP and the time of exposure were varied systematically, showed that optimal effects on the furrowing potency were obtained by immersing the eggs in relatively low concentrations (0.0005 M–0.001 M), starting 25–35 minutes prior to the time when the first cleavages are due to occur. By such treatment the onset of first cleavage was very slightly (2-3 min.) delayed, but the subsequent cleavages closely followed the schedule of the untreated controls and the percentage of normal gastrulae obtained from the ATP-treated eggs was higher, slightly but consistently, than in the controls.

Provided that the immersion started 25 minutes prior to the onset of first cleavage, the time of exposure to ATP was not very critical, since eggs placed in ATP solutions any time within the first half hour after insemination displayed essentially the same behavior. But the concentration factor proved to be very critical. On the higher side, for example, 0.004 M solutions (at 20°C.) gave a 90–95% inhibition of the cleavage of Chaetopterus (60–70% in Arbacia); and 0.002 M solutions gave a 10–15 minute delay in first cleavage, although the eventual development to the gastrula stage was not adversely affected. On the lower side, a lesser improvement of the furrowing potency could be detected at a concentration of 0.00025 M, and scarcely any effect was found at 0.000125 M. In most of the experiments dealing with furrowing potency of the eggs,

therefore, the concentration was fixed at 0.0005 M and the immersion time was between 25 and 40 minutes.

ATP increases in furrowing potency (low temperature experiments). Although the furrowing potency (force exerted by the furrow as it cuts through the egg) cannot be measured directly, relative values may be obtained by determining the point at which the potency first becomes inadequate for successful cleavage when various inhibiting agents (e.g., low temperature or high pressure) are employed.

The low temperature weakening of the furrowing potency of several types of marine eggs, described by Marsland ('50) and by Marsland and Landau ('54) appears to be related to the fact that for each type of egg there is a minimum temperature below which successful cleavage cannot ordinarily occur. For Chaetopterus this minimum displays a rather high value, namely, 18°C. (as reported by Hoadley and Brill, '37, and confirmed in the present experiments), compared to the 9° value found for Arbacia punctulata. In testing the effects of ATP upon the furrowing potency of these eggs, therefore, it was decided to use an experimental temperature 2–3 degrees below the minimum which ordinarily permits successful cleavage, — namely 15°C. for Chaetopterus, and 7°C. for Arbacia.

In these experiments the eggs were fertilized and allowed to develop at room temperature (22°–24°C.) until 15 minutes prior to the time when the first furrows were expected, at which point 2–3 drops of a dense egg suspension were placed in 25 cc. of ATP-sea water solution and in normal sea water, each to the proper low temperature. Thus the total period of immersion in the low temperature environment was 25–30 minutes, since the onset of furrowing in the cooled media was delayed by 10–15 minutes.

Table 1 shows the very definite increase in the furrowing potency which is displayed at low temperature by both kinds of eggs in the presence of rather low concentrations of ATP, under the specified conditions. Apparently, small quantities of ATP available in the outside medium enable the eggs to

overcome the cleavage block imposed by abnormally low temperature — the maximal effect being approached at a concentration of 0.0005 M.

Some variation in the performance of different batches of eggs was found. This is indicated in each case by the range of percentage (table 1), which specifies the upper and lower limits of the variation found in the 3–4 experiments done under each condition.

TABLE 1

ATP-increase in the furrowing strength

KIND OF EGG		PERCENTAGE OF SUCCESSFUL FURROWING (1ST CLEAVAGE)				
		No ATP	ATP in sea water			
			0.000125M	0.00025M	0.00038M	0.0005M
		%	%	%	%	%
Chaetopterus	15°	0–5 [1]	5–10 [2]	15–25	30–40	80–90
	16.5°	0–8 [1]	....	.....	.....	50 [3]
Arbacia	7°	1.5	....	.....	.....	40
		2.0	....	.....	.....	50

[1] In some experiments, a few of the eggs showed incipient polar lobes and shallow furrows, but almost all later reverted to spheres.

[2] Many of these eggs showed distinct furrows, but most of these receded later.

[3] Experiments done late in season. Eggs probably not in optimum condition.

High pressure experiments. A further study of the effects of ATP on the furrowing potency was made in the temperature range where successful cleavage normally occurs, utilizing high pressure to block the furrows. Previous work (Marsland, '50, and Marsland and Landau, '54) has shown that the furrowing potency of various eggs rises with temperature, since higher and higher pressures are required to block furrowing each time the temperature is raised by 5°C. (see figs. 1 and 2). Consequently, it was possible to ascertain whether or not ATP tends to increase the capacity of the egg to overcome the pressure block at temperatures ranging from 10°–30°C.

To avoid excessive delays at lower temperatures (10° and 15°C.) the eggs were fertilized in normal see water and kept at room temperatures until 15–20 minutes prior to the time when, at room temperature, incipient furrows were to be expected. Then 2–3 drops of a dense suspension were added to 25 cc of the chilled experimental solution (either 0.0005 M ATP or normal sea water), whereupon the eggs were immediately put into the microscope-pressure chamber, which already was equilibrated to the proper low temperature in the thermostated housing. The objective was to expose the eggs to the cold environment (with or without ATP) for 30 minutes prior to the onset of furrowing (which was observed directly) and prior to the application of the pressure, — and in all acceptable experiments the variation in timing did not exceed 5 minutes. On the other hand, at higher temperatures (20°–30°C.) the eggs were fertilized and kept at the experimental temperature for the full period between insemination and first cleavage, although usually they were not put into the ATP medium until 30 minutes prior to the appearance of incipient furrows.

Timing the application of pressure proved to be a rather critical matter, since shallow furrows, compared to deeper ones, can be blocked by somewhat lesser pressures. Accordingly, the eggs were kept under continuous observation as the time of furrowing approached. For Arbacia the pressure was built up as soon as an estimated 50% of the eggs in a chosen field displayed perceptible furrows; and for Chaetopterus, the compression was started when about 50% showed at least incipient polar lobes.

The results of these experiments are plotted in figures 1 and 2. Here it may be seen that ATP, available at low concentration (0.0005M) in the surrounding sea water, effects a definite increase in the furrowing potency in both kinds of eggs under widely varying conditions of temperature and pressure. Specifically, the furrowing of the Chaetopterus egg, which ordinarily is blocked by pressures of 2000, 2500 and 3000 lbs./in.^{2} at 20°, 25° and 30°C. respectively, successfully achieves first cleavage when additional ATP is provided (fig.

1 and table 2). And similarly, when extra ATP is available, successful cleavage occurs in the Arbacia egg under pressures of 3000, 4000, 5000, and 6000 lbs./in.^{2}, at 10°, 15°, and 20°, and 25°C. respectively, whereas under similar conditions, without ATP, the furrowing fails (fig. 2 and table 3). In fact, under each of the different pressure-temperature conditions, in both

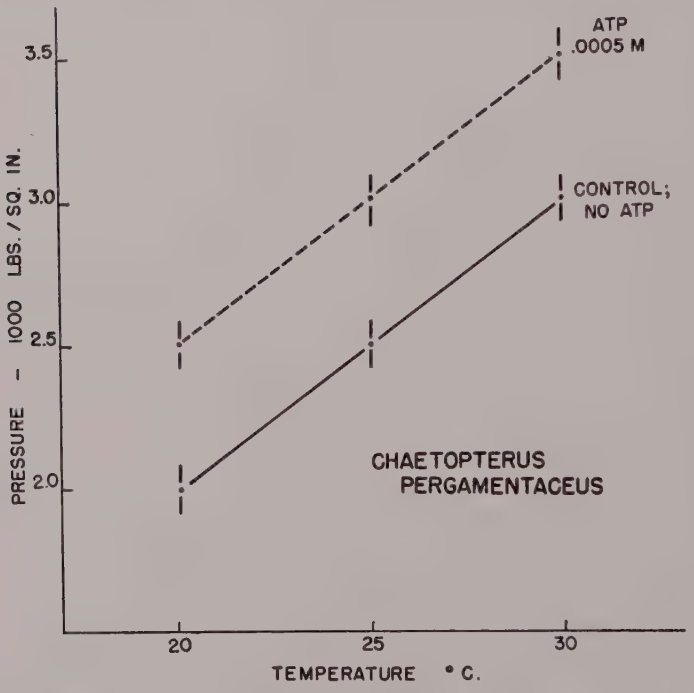

Fig. 1 Effects of ATP on the furrowing strength of Chaetopterus eggs. Minimum pressure required to block furrowing at different temperatures.

kinds of eggs, the pressure level required to establish a cleavage block displayed a consistent increase of 500 lbs./in.^{2}.

Since the minimum pressure required to block furrowing at a given temperature tends to be less if the pressure is applied before furrowing starts and tends to increase if the pressure is established later, as the furrows grow deeper and deeper, and since all eggs in a given batch do not start furrowing in exact synchrony, particular attention must be

given to such eggs as can be observed directly at the time when the pressure is built up. In our pressure chamber only 10–15 eggs, on the average, can be brought into each microscopic field. Consequently, applying pressure at the time when 50% of the observed eggs displayed definite furrows allowed a small percentage of the eggs in the chamber as a

TABLE 2

Percentage of successful furrowing, with and without ATP, under various temperature-pressure conditions. (Eggs of Chaetopterus pergamentaceus)

TEMPERATURE °C.	PRESSURE LBS./IN.²	ATP (.0005M)	NO ATP	SPECIAL CONDITION OR COMMENT
		%	%	
20°	2000	85	10	In ATP 15 min. Blisters appear at furrowing time; disappear later.
		50	10	In ATP only 14 min. Pressure applied very early.
		87	< 10	In ATP 53 min.
	2500	10	0	In ATP 23 min.
		14	0	In ATP 15 min.
25°	2500	60	5	In ATP 13 min.
		85	0	In ATP 25 min.
		90	0	In ATP 40 min.
	3000	0	0	
		6	0	
30°	3000	80	0	In ATP 31 min.
	3500	1	0	In ATP 31 min.

whole (those with exceptionally advanced furrows) to complete their cleavage, even when the pressure, on the basis of the behavior of the observed eggs, was judged to be adequate to block furrowing. And similarly, pressures which were judged to be not adequate to block furrowing did give a block in 40–50% of such eggs as had not started the furrowing process at the time when the pressure was applied.

Generally speaking the behavior of the eggs in the observed field and in the chamber as a whole (determined by surveying many fields 20 minutes after establishing the pressure) showed a close correspondence. However, since timing of the pressure treatment was known precisely for only a limited number of eggs, at least three experiments were performed for each of the different temperature-pressure combinations.

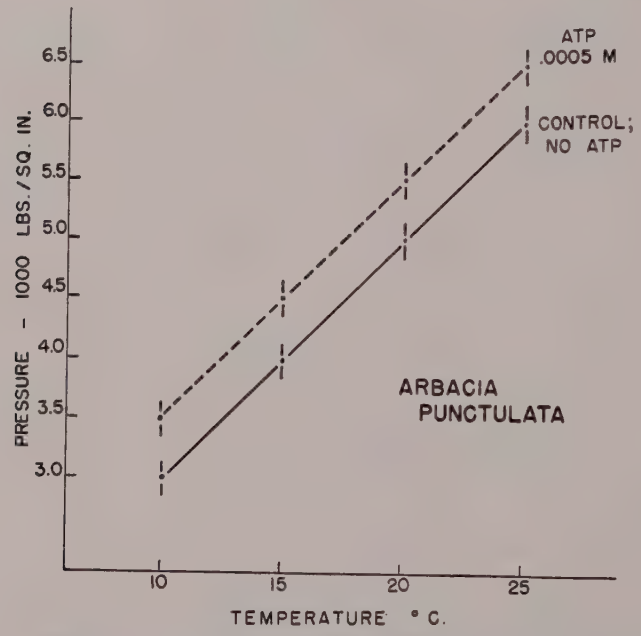

Fig. 2 Effects of ATP on the furrowing strength of Arbacia eggs. Minimum pressure required to block furrowing at different temperatures.

Also it must be recorded that a few batches of eggs were found, especially toward the end of the breeding season, in which the furrowing strength of the control samples was lower than is indicated in figures 1 and 2. However, in all such cases, the furrowing performance of ATP-treated samples was measurably better than that of untreated controls.

Effects of related compounds. It seemed important to determine whether or not the high energy phosphate moiety of the ATP molecule is essential in regard to the action

on the furrowing potency. Accordingly, parallel experiments were carried out using adenosine-5-monophosphate (AMP), adenosine, and dibasic sodium phosphate (Na_2HPO_4) in place of ATP. Both Chaetopterus and Arbacia eggs were used in these experiments, but only one temperature, namely 25°C. for Chaetopterus and 20°C. for Arbacia was covered.

TABLE 3

Percentage of successful furrowing, with and without ATP, under various temperature-pressure conditions. (Eggs of Arbacia punctulata)

TEMPERATURE °C.	PRESSURE LBS./IN.²	ATP (.0005M)	NO ATP	SPECIAL CONDITION OR COMMENT
		%	%	
10°	2500	95	1	Fertilized egg placed in experimental media 45 minutes prior to expected time of furrowing.
	3000	5	0	
	3500	0	0	
15°	3500	70	5	In ATP only 25 min. prior to furrowing.
	4000	85	2	In ATP 37 min.
	4500	6	0	
20°	5000	80	8	In ATP 57 min.
		75	5	In ATP 68 min.
		75	0	0.001M ATP used; in ATP 53 min.
	5500	5	0	In ATP 56 min. Many furrows start but few finish.
25°	6000	80	0	100% of those which showed shallow furrows when pressure was applied or developed furrows soon after furrowed successfully in ATP.
	6500	0	0	

At a concentration of 0.0005 M, which gave maximal effects for ATP, none of the other substances (AMP, adenosine and Na_2HPO_4) gave any measurable effect upon the furrowing potency — as judged by the minimum pressure required to block first cleavage. At a concentration 4 times higher, namely, 0.002 M, the AMP appeared to give a slight improvement in the percentage of complete cleavages in some

batches of eggs but not in others; and even in the favorable cases, the difference between the AMP-treated and the untreated samples was just barely significant statistically. Nevertheless, the higher concentration of AMP seemed to have a stabilizing effect upon the cleavage furrow. These furrows tended to persist (not recede) despite the high pressure, even when they did not advance and complete the cleavage. Adenosine gave absolutely no effect in concentrations up to 0.016 M. At all concentrations used, the Na_2HPO_4 in sea water gave a definite precipitation, and no improvement of the furrowing performance in Chaetopterus was found at any concentration. In fact, higher concentrations (0.002 M and above) seemed to give an adverse effect upon the furrowing strength. However, in Arbacia, 0.002 M Na_2HPO_4 gave a slightly enhanced furrowing potency which approached the effects of AMP.

ATP effects on gel structure. To test the hypothesis that the furrowing potency is determined by the gelational state of the cytoplasm bordering the cleavage furrow, it was necessary to ascertain whether or not the ATP treatment induces parallel changes in the gel structure of this cortical layer. Accordingly, centrifugal measurements were undertaken at temperatures from 10° to 30°C. and at pressures ranging up to 8000 lbs./in.^{2} Only the eggs of Arbacia were used in these experiments, since Chaetopterus eggs undergo fragmentation in relatively weak centrifugal fields, especially when high pressures are applied, making it difficult to determine the centrifugal displacement of the cortical granules. Moreover, the measurements were made on the unfertilized eggs of Arbacia in which a displacement of the cortically embedded pigment bodies occurs much more readily than later, when the fertilized egg is about to begin furrowing. Such measurements are pertinent, however, since it has been shown (Marsland, '38, '39a and '50) that the cortical gel in unfertilized and cleaving eggs, being of the same essential type, displays proportional changes of structure throughout a wide range of temperature and pressure variation, although, in absolute

terms, the gelational strength in the cleaving egg is of a much higher order.

Soon after these experiments were started it was found that sharper differences between ATP-treated and untreated eggs were obtained when the ATP concentration was increased to 0.001 M (as compared to the 0.0005 M solutions used in determining the furrowing potency). However, the time of immersion (25 minutes) in ATP used in most of the other experiments proved adequate for maximal effects on the gel structure and this period was employed throughout. The standard procedure was, accordingly, to equilibrate the eggs at the experimental temperature and then to transfer samples to the 0.001 M ATP in sea water (likewise equilibrated to the proper temperature) exactly 25 minutes prior to each centrifugation. The centrifuge speed, regularly checked stroboscopically, was standardized at 7250 rpm, giving a uniform force of 4000 $\times$ g. In each experiment, control eggs (without ATP) were simultaneously centrifuged and matched against the experimental sample. The displacement end-point was the same as previously described (Marsland and Landau, '54).

ATP effects on gel strength; temperature experiments. As may be seen in figure 3, these experiments show that ATP consistently and definitely does have a strengthening effect upon the gelational state of the cortical cytoplasm of the Arbacia egg throughout the whole normal temperature range (10°–30°C.) of the species. In fact, the strengthening of the gel structure by ATP and temperature appears to represent an additive phenomenon, so that the ATP curve, although higher in position, displays the same slope. In relative terms, moreover, the fortifying action of ATP under the specified conditions appears to be almost as great as that produced upon the gel structure when the temperature of the system is raised by 5°C.

Pressure experiments. The capacity of ATP to counteract the pressure furrow block makes it important to determine

how this compound may modify the well-known weakening effects of higher pressures on protoplasmic gel structures generally. These experiments involved spinning treated and untreated egg samples in the pressurized compartment of the pressure-centrifuge and determining the time, at each pressure level, required to give the standardized degree of displacement of the cortically embedded pigment bodies. The

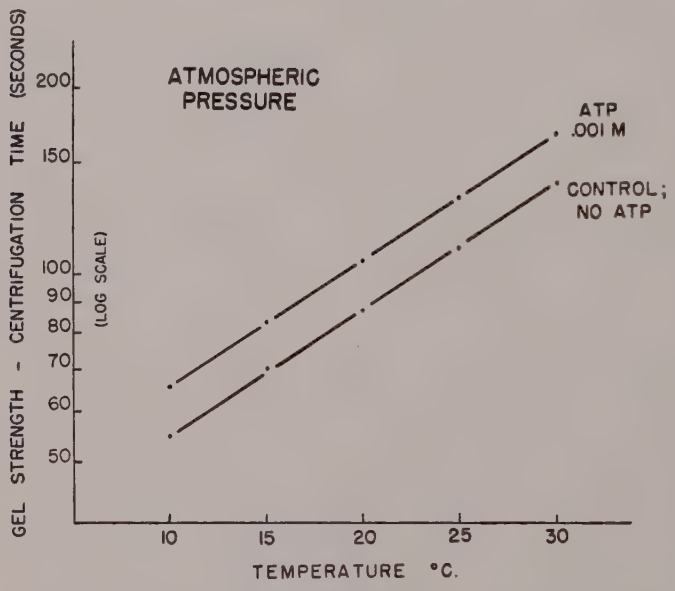

Fig. 3 Effects of ATP on the gelational state of the cortical cytoplasm of Arbacia eggs. Gel strength plotted as a function of temperature.

measurements were carried out at only one temperature, namely 25°C., since previous experiments (Marsland, '50) have shown that changing the temperature merely shifts the position of the pressure-gel strength curve without affecting its slope.

The results of these experiments are plotted in figure 4. Here it may be seen that the ATP-treated gel system displays the same exponential weakening of its structural strength with rising pressure as does the untreated system,

but that the gel strength values are definitely and consistently higher in the ATP-treated system, throughout the whole range of pressures employed. In both systems the pressure effect is drastic, so that the gel strength values obtained at 8000 lbs./in.^{2} are only about 14% of the initial atmospheric

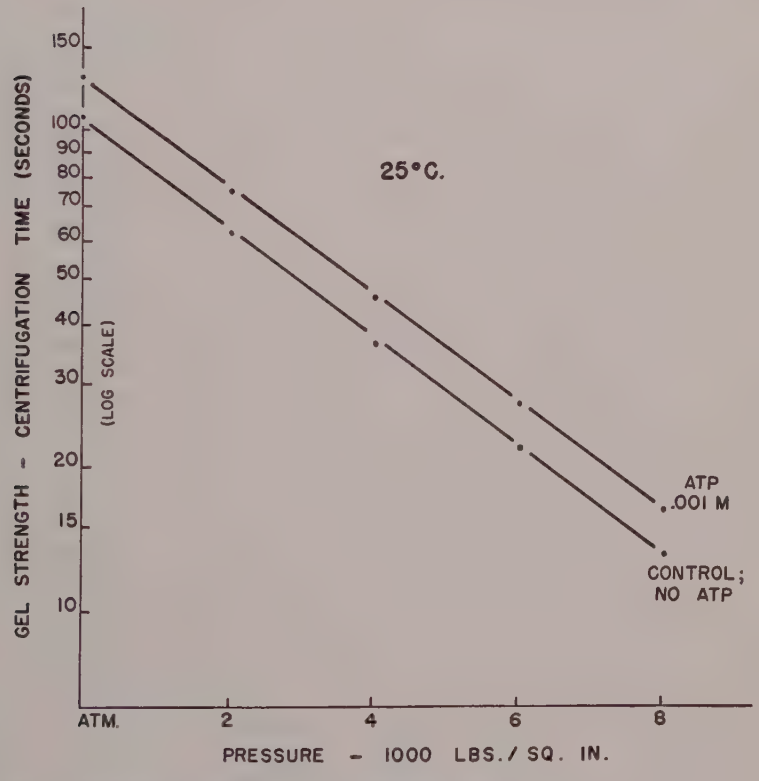

Fig. 4 Effects of ATP on the gelational state of the cortical cytoplasm of Arbacia eggs. Gel strength as a function of pressure, at 25°C.

values, but the differential in the values is plain throughout. In terms of pressure, this differential represents approximately 500 lbs./in.^{2}, since the gel strength values for treated and untreated eggs tends to be equal when the pressure to which the ATP eggs are treated is 500 lbs./in.^{2} higher than in the untreated specimens.

DISCUSSION

Generally speaking, it seems evident that the experiments provide further support for the cortical gel contraction theory of cytokinesis. This is especially true in regard to the primary postulate, namely, that the furrowing potency of the animal cells is determined by the structural state of the peripheral layer of gellated cytoplasm in the region bordering the cleavage furrow. Under widely varying experimental conditions, whenever the gelational state of this cortical cytoplasm is weakened — by lower temperature or by higher pressure, there is a corresponding fall in the furrowing strength, and conversely, whenever structural strength of the cell cortex is fortified, by higher temperature or lower pressure or by ATP added to the medium, the furrowing strength displays a corresponding increase.

The endothermic nature of protoplasmic gelation requires that there be a basic pattern of metabolism in the cell which determines when and where gel structures are to be formed. The present experiments indicate that the ATP system may play an important role in this metabolism, but precisely how is an open question. The formation of a gel structure presumably involves the interlinking of unfolded protein molecules (or aggregates) and the contraction of such a gel seems to represent a rapid sort of syneresis whereby the protein components of the gel network undergo a forcible folding without relinquishing the intermolecular linkages. But whether the large fund of energy liberated by the hydrolysis of ATP serves to strengthen the intermolecular linkages or to reinforce the folding tendency of the protein units or to fulfill some other function cannot be said at present. It seems evident that gelated parts of the protoplasm are potentially contractile, which enables relatively undifferentiated cells to perform mechanical work, but as in the case of muscular contraction, the precise role of the ATP system in the contraction phenomenon is still obscure.

Theoretically, it is difficult to understand how a large polar type of molecule, such as ATP, can gain entrance into the cell

from the surrounding medium, but it cannot be denied, in view of these and other experiments, that ATP administered via the environmental solution does have a marked influence on the behavior of the cells. The high energy moiety of the ATP is evidently quite important to such an action, since AMP, adenosine and inorganic phosphate used in equivalent concentration, are not effective. However, the possibility remains that ATP, as such, does not enter the cell. In fact, it has been suggested by Lindberg ('50) and by Runnstrom and Kriszat ('50) that ATP merely transfers its labile phosphate across the surface membrane to the subjacent layer of cytoplasm.

SUMMARY

1. Adenosine triphosphate (ATP), used at low (0.0005 M) concentration in the surrounding sea water, definitely increases the furrowing strength of Arbacia and Chaetopterus eggs, permitting them to cleave successfully under a variety of temperature-pressure conditions which normally block the furrows.

2. At low temperatures, the furrowing of Arbacia and Chaetopterus eggs, which normally is blocked at 9° and 18°C., respectively, is not blocked in the presence of 0.0005 M ATP. In fact, ATP reduces the blocking temperature to 7°C. for Arbacia and to 15°C. for Chaetopterus.

3. Likewise the minimum pressure required to block cleavage at different temperatures is definitely increased. Specifically, the furrowing of the Chaetopterus egg which ordinarily is blocked at 2000, 2500, and 3000 lbs./in.^{2} at 20°, 25° and 30°C., respectively, requires 500 lbs. additional pressure at each temperature when the eggs have been immersed in 0.0005 M ATP. Similarly for ATP-treated Arbacia eggs, the minimum furrow-blocking pressure is increased from 3000 to 3500 lbs./in.^{2} at 10°C; 4000 to 4500 lbs./in.^{2} at 15°C.; 5000 to 5500 lbs./in.^{2} at 20°C.; and 6000 to 6500 lbs./in.^{2} at 25°C.

4. Equivalent concentrations of adenosine-5-monophosphate, adenosine and dibasic sodium phosphate, used in place

of ATP, had no effect upon the furrowing strength. However, both of the phosphates but not the adenosine, gave a slight increase when fourfold higher concentrations were employed.

5. Centrifugal measurements of the gelational state of the cortical cytoplasm of the unfertilized Arbacia egg gave significantly higher values for ATP-treated vs. non-treated eggs — throughout the ranges of temperature (10°–30°C.) and pressure (up to 8,000 lbs./in.^{2}) which were employed.

6. The data are interpreted in terms of the gel contraction hypothesis, on the assumption that part of the high energy of the labile phosphate groups can be utilized by the cell in the (endothermic) formation of its essential gel structures.

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